DEFINING TET ENZYME REQUIREMENTS IN NORMAL VERTEBRATE DEVELOPMENT AND HEMATOPOIESIS

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate

School of Medical Sciences

in Partial Fulfillment of the Requirements for the Degree

of Doctor of Philosophy

By

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December 2017

DEFINING TET ENZYME REQUIREMENTS IN NORMAL VERTEBRATE DEVELOPMENT AND HEMATOPOIESIS

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Cornell University, 2017

The ten-eleven translocation proteins TET1, TET2, and TET3 mediate oxidation of the modified base 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) through an iterative process. Oxidation of 5mC has been suggested to play the first step in DNA demethylation pathways. However, the functions of TET proteins in promoting vertebrate development and differentiation have not been fully defined. To systematically assess the requirements for TET proteins during development, we mutated the zebrafish orthologs of *TET1, TET2,* and *TET3,* and examined single, double and triple mutant genotypes. Using these mutant lines, we identified Tet2 and Tet3 as the major 5-methylcytosine dioxygenases in the zebrafish embryo. In addition, we have uncovered overlapping requirements for Tet2 and Tet3 in hematopoietic stem cell (HSC) development. Our results identify Tet2 and Tet3 as early regulators of the notch signaling required for induction of the hematopoietic transcription factor program in the hemogenic endothelium. These studies have also revealed a potential link between inflammatory signaling from the primitive hematopoietic system and defective notch signaling in tet2/3 mutants. Further study revealed that enhanced Tet1 activity could compensate

the loss for Tet2/3 during HSC emergence, providing evidence that members of TET family behave similarly and can act interchangeably when expressed at sufficient levels. Lastly, we discuss applications of the *tet2/3*^{DM} platform to identifying novel regulators of TET proteins. Collectively, this research defines essential, overlapping functions for TET proteins during embryonic development and uncovers a requirement for 5hmC in regulating HSC emergence. Additionally, the findings, as well as the *tet2/3* double mutant larvae we generated, hold great promise to facilitate the study of blood cancer treatment.

BIOGRAPHICAL SKETCH

Cheng Li was born and grew up in Beijing, China. He graduated from Beijing No.2 Middle School in 2006 and entered the Hong Kong Polytechnic University in the same year. During his early years in the college, he developed a keen interest in biological sciences and transferred his studies from architecture to biology. He earned his bachelor degree in Applied Biology and Biotechnology with first honor on Dean's list in 2011. In the fall of 2011, Cheng came to New York City and became a graduate student of the BCMB Allied program at Weill Cornell Graduate School of Medical Sciences. In the summer of 2012, Cheng started his thesis work in Dr. Mary Goll's lab at the developmental biology department of Sloan Kettering Institute to investigate how epigenetic factors regulate the development of hematopoietic stem cells using zebrafish as the system.

ACKNOWLEDGEMENTS

I would like to thank my Ph.D. advisor Dr. Mary Goll for being a great mentor. My thesis work could not be done without her support and mentorship. I will be always grateful that Mary offered me the opportunity to do the most exciting science in the lab.

I would also like to thank lab members in Mary's lab. They created a fun and collaborative environment for the work, and I will miss them a lot.

I appreciate the support and critiques I received from my committee members Dr. Todd Evans, Dr. Danwei Huangfu, my former committee member Dr. Mary Donohoe, and the chairperson of my thesis defense Dr. Yariv Houvras. They are the witnesses of my growth to become a scientist.

Finally, I want to thank all the love I get from my friends and my family, especially my parents Linzhuo Li and Yuqin Wei, and my wife Yichen Hua, who are the ultimate source of me.

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CHAPTER ONE: INTRODUCTION

Section 1. Oxidation of 5-methylcytosine by the ten-eleven translocation (TET) proteins

1.1 5-methylctyosine

Methylation of DNA at the 5' position of the cytosine ring (5mC) represents a conserved modification contributing to transcriptional repression. This modification is essential for normal vertebrate development (Goll & Bestor, 2005). DNA methylation has critical roles in controlling gene expression, ensuring X-chromosome inactivation and reinforcing genomic imprinting (Jaenisch & Bird, 2003; Smith & Meissner, 2013). Aberrant DNA methylation is also observed in many diseases including cancer. In vertebrates, DNA methylation is primarily restricted to symmetrical CpG dinucleotides, which are extensively methylated across the genome. An exception is CpG islands, which are typically unmethylated. These CpG islands represent less than 10% of the total genomic CpG content and are prevalent at transcription start sites of developmentally regulated genes (Smith & Meissner, 2013). Abnormal gains in 5mC in normally unmethylated CpG islands have been linked to the inactivation of tumor suppressor genes in many cancers (Zhu, Wang, & Qian, 2016). However, the mechanism by which 5mC becomes abnormally accumulated at the regulatory regions in cancers remains unclear.

A better understanding of how 5mC is regulated is of particular importance in understanding normal development and the pathogenesis of diseases.

The DNA methylation reaction is executed by two groups of DNA methyltransferases: the maintenance methyltransferase, DNMT1, and the de novo methyltransferases of the DNMT3 family (Denis, Ndlovu, & Fuks, 2011). De novo methyltransferases methylate unmodified DNA to establish methylation patterns. During maintenance of DNA methylation, the cofactor UHRF1 recruits DNMT1 to hemimethylated CpG dinucleotides at the replication fork, allowing faithful propagation of methylation patterns to the newly synthesized DNA strand (Bostick et al., 2007; Sharif et al., 2007). The orchestration of de novo and maintenance DNA methylation enables the heritability of 5mC patterns throughout the development of the organism.

1.2 TET proteins catalyze oxidation of 5-methylcytosine

For many years, 5mC was thought to be the only modified DNA base in the mammalian genome. However, in 2009, two groups reported the presence of the 5-hydroxymethylcytosine (5hmC), an oxidative derivative of 5mC, in mammalian DNA (Kriaucionis & Heintz, 2009; Tahiliani et al., 2009). Subsequent studies have demonstrated that the ten-eleven translocation proteins (TET1, TET2 and TET3) oxidize 5mC to 5hmC and that they are capable of further oxidation of 5hmC to 5-formylcytosine (5fC) and 5-

carboxylcytosine (5caC) through an iterative process (Tahiliani et al., 2009; H. Wu & Zhang, 2011). These novel modified bases may represent new epigenetic states in the genome. Insights into the functions of the 5mC-oxidized products and how TET proteins regulate the oxidation process will greatly enhance our understanding of epigenetic regulation of normal development and diseases.

1.3 TET protein structure and activity

TET1 was first identified as a fusion partner of mixed-lineage leukemia (MLL), resulting from the chromosomal translocation t(10;11)(q22;23) in acute leukemia (Lorsbach et al., 2003; Ono et al., 2002). TET proteins have been subsequently identified as a family of large multidomain enzymes containing a conserved double-stranded β-helix (DSBH) domain, a cysteine-rich domain and cofactors Fe (II) and 2-oxoglutarate (2-OG) binding domains. Mammalian genomes encode for three *TET* genes (*TET1*, *TET2* and *TET3*). In addition to the catalytic domain, TET1 and TET3 contain an N-terminal CXXC zinc finger domain, allowing them to bind directly to DNA sequences (H. Wu & Zhang, 2011) (Figure 1). In the case of TET2, the CXXC domain has become a separated protein named IDAX as the result of a chromosomal inversion event (Ko et al., 2013).

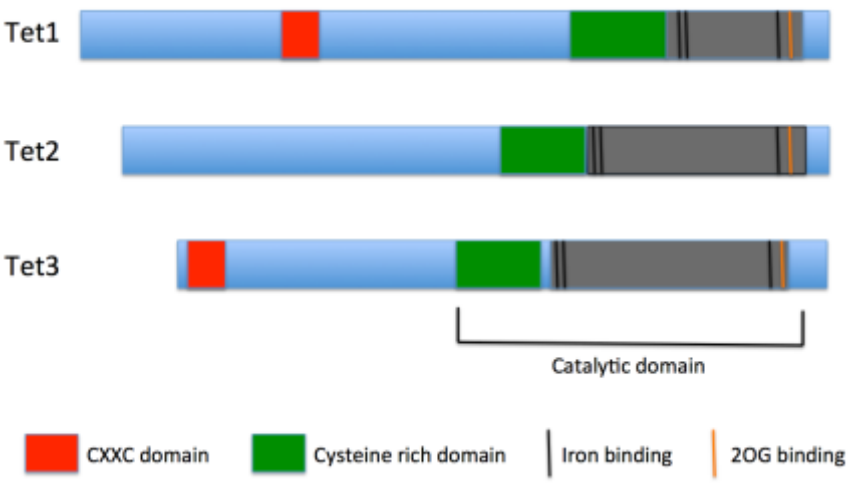


Figure 1. Domain architecture of mammalian Tet1, Tet2, and Tet3 proteins. Four conserved domains—including CXXC zinc finger domain (red), the cysteine-rich domain (green), the double-stranded β-helix (DSBH) fold (grey) of the 2-OG binding domain (orange) and Fe(II) binding domain (black)—are indicated. Tet2 does not contain a predicted CXXC domain.

1.4 Regulators of TET activity and function

Mounting evidence suggests that TET protein activity and recruitment are regulated at multiple levels. For instance, TET enzymatic activity can be stimulated by specific metabolites, cofactors, and post-translational modifications. Additionally, interaction with binding partners is likely to affect TET protein localization as well as protein stability.

Vitamin C is one cofactor that positively regulates TET activity. Vitamin C has been shown to stimulate TET enzymatic activity in various cultured cells with increased levels of the cytosine oxidation products (Blaschke et al., 2013; Gustafson et al., 2015; Sasidharan Nair, Song, & Oh, 2016; Yue et al., 2016). Molecular study indicates that Vitamin C interacts directly with the catalytic

domain of TET proteins and provides a local reducing environment that increases recycling efficiency of the Fe (II) cofactor (Yin et al., 2013).

The availability of key metabolites such as 2-OG is also important for TET activity. Mutations in genes coding isocitrate dehydrogenases 1 and 2 (IDH1/2), succinate dehydrogenase (SDH), and fumarate hydratase (FH) have been reported in various types of cancers (Oermann, Wu, Guan, & Xiong, 2012). These mutations can result in the production of metabolites such as 2-hydroxyglutarate (2-HG) that compete with 2-OG. The accumulation of these metabolites has been shown to inhibit the enzymatic activity of TET proteins.

TET proteins are also modified post-translationally. For example, TET proteins can bind to and be monoubiquitylated by the E3 ubiquitin ligase CRL4 (VprBP) on a highly conserved lysine residue at TET2 K1299 (Nakagawa et al., 2015). This monoubiquitylation promotes TET protein binding to chromatin. Tet proteins have also been shown to undergo massive phosphorylation and O-GlcNAcylation at the N terminus and in the low-complexity insert region. These two modifications appear to act in a competitive manner (Bauer et al., 2015). These modifications have been suggested to allow for rapid modulation of TET protein localization and protein stability in response to different environmental stimuli.

1.5 Genome distribution of 5hmC, 5fC and 5caC

While 5mC represents 4% to 5% of total cytosine, its oxidized form, 5hmC ranges from 0.1% to 0.5% of total cytosine and is highly variable across different tissues (Rasmussen & Helin, 2016). The products of further TET-mediated oxidation, 5fC, and 5fC, are observed at 10-fold and 100-fold lower than the abundance of 5hmC (Ito et al., 2011; Kriaucionis & Heintz, 2009; Szwagierczak et al., 2010).

Mapping of the cytosine oxidation products has been performed in a number of tissues (Pastor et al., 2011; Szulwach et al., 2011; Williams et al., 2011; H. Wu et al., 2011; Xu et al., 2011). In ESCs, 5hmC enrichment was found at distal regulatory regions, gene bodies and intragenic regions with low CpG content, similarly, 5fC and 5caC were found mostly at distal regulatory regions but at a significantly lower amount compared with 5hmC (Pastor et al., 2011; Williams et al., 2011).

1.6 Potential roles for TET enzymes in mediating DNA demethylation

Mechanistically, it has been suggested that conversion of 5mC to 5hmC by TET enzymes may provide a first step in long-sought-after DNA demethylation pathways. Both active and passive mechanisms have been proposed.

Passive demethylation refers to the failure to maintain DNA modification patterns during DNA replication process (Figure 2). During DNA replication, unmodified cytosine is incorporated into the newly synthesized strand, creating a hemi-modified CpG dinucleotide. A 5mC:C site is recognized by UHRF1, which helps to recruit DNMT1 to the hemi-5mC site. However, in vitro studies have shown that DNMT1 activity is reduced on a substrate DNA containing 5mC-oxidized products (Hashimoto et al., 2012). In this manner, 5hmC-, 5fC-, and 5caC-modified CpG sites may promote demethylation through multiple rounds of DNA replication.

In contrast, active DNA demethylation via 5mC oxidation has been suggested to involve base excision repair (BER) pathways (Figure 2). In particular, studies have implicated thymine DNA glycosylase (TDG) in this process. The TDG enzyme recognizes and excises mismatch in G:U and G:T base pairs, leaving abasic sites that can be subsequently detected and repaired by BER pathway (H. Wu & Zhang, 2014). In addition to these mismatches, TDG exhibits significant enzymatic activity toward 5fC and 5caC but not 5hmC in vitro (He et al., 2011; Spruijt et al., 2013). Based on these studies, an active DNA demethylation pathway has been proposed in which 5fC and 5caC generated by TET proteins from 5mC are excised by TDG, the abasic site is then replaced by an unmodified cytosine through BER pathway. The net outcome of this process is the reversal of 5mC to unmodified cytosine. Consistent with this model, studies have shown that deletion of *Tdg* in mouse

ESCs results in significant increase in 5fC and 5caC levels (He et al., 2011; Shen et al., 2013); conversely, overexpressing TDG in HEK293 cells leads to depleted 5fC and 5caC levels (Nabel et al., 2012). Taken together, the in vitro and in vivo data suggest TDG/BER-dependent pathway is at least one of the TET-mediated active DNA demethylation processes.

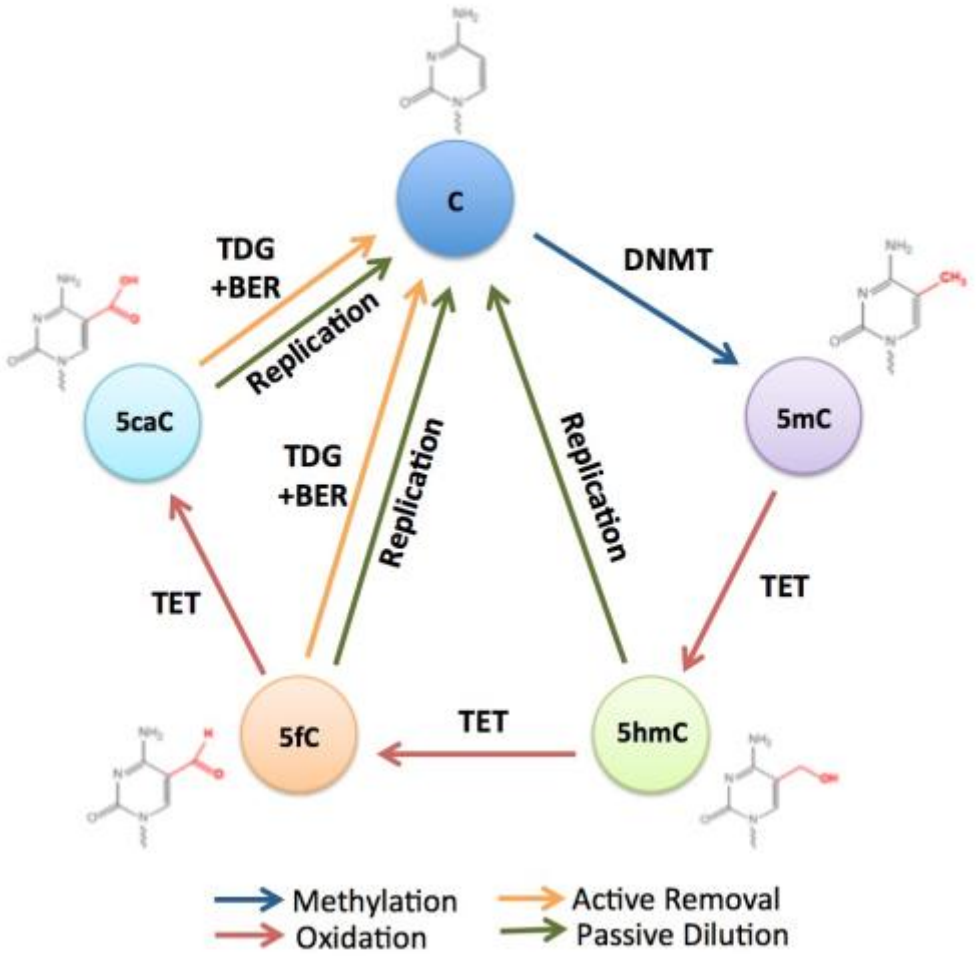


Figure 2. Mechanisms of passive and active DNA demethylation. DNA methyltransferases (DNMTs) convert unmodified cytosine to 5-methylcytosine (5mC). 5mC can be converted back to unmodified cytosine by TET-mediated oxidation to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), followed by excision of 5fC or 5caC mediated by thymine DNA glycosylase (TDG) coupled with base excision repair (BER) Figure based on Wu & Zhang, 2017.

1.7 Additional functions for 5mC-oxidized products

Given their stability and abundance, it is tempting to speculate that the 5mC-oxidized products have additional functions beyond their roles in promoting DNA demethylation. Indeed, recent findings using isotope labeling have revealed that 5hmC and 5fC are rather stable (Bachman et al., 2015, 2014). These results imply that 5hmC and 5fC can be uncoupled from active DNA demethylation pathways and may exhibit additional functions. In support of this hypothesis, analysis of TET activity has demonstrated that the rate of cytosine oxidation is significantly reduced for the 5hmC and 5fC substrates compared with 5mC substrate (Hu et al., 2013), this differential substrate preference can be attributed to subtle differences in the catalytic activity of the TET enzyme (Hu et al., 2015). It is, therefore, plausible that the intrinsic properties of TET enzymes restrain the oxidation process to form 5fC and 5caC, leading to accumulated 5hmC in the genome that is independent of the active DNA demethylation pathway.

Despite substantial efforts, only a few readers of 5hmC have been identified to date (Iurlaro et al., 2013; Song & Pfeifer, 2016). One confirmed 5hmC reader, UHRF2, exhibits specific binding activity to fully hydroxymethylated or hemi-hydroxymethylated DNA sequences over methylated DNA sequences

(Spruijt et al., 2013; Zhou et al., 2014). However, the biological significance of the binding of UHRF2 and 5hmC remains to be explored. Alternatively, some evidence suggests 5hmC might act to block binding of other proteins in some scenarios. For example, proteins that bind methylated DNA, such as MBD1, MBD2, and MBD4 exhibit lower binding affinity when oxidized 5mC products exist in the DNA substrates (Hashimoto et al., 2012; Jin et al., 2010).

In contrast to 5hmC, studies have revealed a number of proteins that are able to bind to 5fC and 5caC (Spruijt et al., 2013; Iurlaro et al., 2013), suggesting these 5mC-oxidized products might encode additional epigenetic information. For example, RNA polymerase II (Pol II) has been shown to interact with both 5fC and 5caC in vitro (Kellinger et al., 2012). Structural studies indicate that the interaction of Pol II and 5caC induces a positional shift of Pol II, which is unfavorable to the addition of nucleotides to the RNA strand (Wang et al., 2015). The study suggests the interaction of Pol II and 5caC may regulate the elongation speed of genes with 5fC and 5caC within the gene bodies. Further in vivo analysis should help to elucidate the biological function of such interactions at the physiological level.

1.8 Tissue specific expression of Tet genes in mammals

In mammals, TET enzymes exhibit distinctive expression patterns in different tissues. For instance, mouse *Tet1* and *Tet2* are highly expressed in

embryonic stem cells (ESCs). However, upon differentiation, *Tet1* becomes down-regulated while *Tet3* is up-regulated. In oocytes, *Tet1* and *Tet2* are minimally expressed while Tet3 contributes to the early 5hmC synthesis in the zygote. *Tet2* and *Tet3* also exhibit high expression levels in a wide spectrum of adult tissues. *Tet2* is the most highly expressed Tet family member in spleen and liver, while the expression level of *Tet3* is the highest in tissues such as cerebellum and cortex. The expression of *Tet1*, in contrast, is relatively low in adult tissues (Szwagierczak et al., 2010).

1.9 Mutation of Tet enzymes in mouse

Individually, homozygous mutation of *Tet1*, *Tet2* or *Tet3* is compatible with mouse embryonic development, although *Tet3* mutant mice die perinatally (Kohli and Zhang, 2013). Conditional deletion of *Tet3* in oocytes results in delayed demethylation of the paternal genome and increased developmental failure, but viable pups can be recovered (Gu et al., 2011), the sub-lethality phenotype is likely caused by *Tet3* haploinsufficiency (Inoue et al., 2015).

In contrast to single mutants, combinatory loss of Tet1 and Tet2 in mice leads to a wide spectrum of defects, including exencephaly, growth retardation, and compromised imprinting. However, some mice survive and develop normally (Dawlaty et al., 2013). Embryos lacking *Tet1* and *Tet3* display abnormal embryonic phenotypes with increased transcriptome variability (Kang et al.,

2015). Compared to single mutants, the complexity of the phenotypes in *Tet1/2* and *Tet1/3* double mutants strongly suggests that combinatory loss of Tet proteins is likely to result in epigenetic abnormalities that increase developmental stochasticity and compromise embryogenesis at multiple levels. To date, whole animal *Tet2/3* double mutant mice have not been described. However, inactivation of all three *Tet* genes in mice leads to gastrulation phenotypes, including primitive streak patterning defects in association with impaired body plan formation. This phenotype has been attributed to hypermethylation of the *Lefty* gene in the absence of functional TET enzymes (Dai et al., 2016). Additionally, *Tet1/2/3* triple-knockout ESCs show impaired differentiation and contribute poorly to chimeras (Dawlaty et al., 2014). All together, these studies suggest that TET family members have overlapping functions in promoting embryonic development.

1.10 TET proteins in hematopoiesis and blood cancers

TET proteins are particularly important to hematopoiesis and mutations in TET genes have been link to various types of blood cancers. In hematopoietic tissues, TET2 is the most abundant TET protein and genetic knockout of *Tet2* leads to increased hematopoietic stem cell (HSC) pool with skewed differentiation capability toward the myelomonocytic lineage in mice (Ko et al., 2015, 2011; Z. Li et al., 2011; Moran-Crusio et al., 2011). Similarly, depletion of *Tet1* results in an overall lymphoid bias and impaired B-cell lineage

differentiation (Cimmino et al., 2015). Mutation of *Tet3* causes minor decreases in the absolute number of HSCs in the mouse bone marrow, while numbers of myeloid, erythroid and B lymphoid cells were unaffected (Ko et al., 2015). In addition, concomitant loss of Tet2 and Tet3 in mice at early B cell stage blocks the pro- to pre-B cell transition in the bone marrow (Lio et al., 2016). Overall, these data show that TET proteins are critical in controlling the differentiation and proliferation of HSCs and pro-B cells.

Mutations in TET proteins have also been observed in a wide range of hematological disorders. TET1 was first identified as a fusion partner of MLL in acute myeloid leukemia and has essential oncogenic roles in some MLL-rearranged leukemias (Huang et al., 2013; Lorsbach et al., 2003). Alterations in *TET2*, on the other hand, are correlated with a wider range of hematological diseases. *TET2* mutations have been reported in patients with myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML), acute myeloid leukemia (AML), myeloproliferative neoplasms (MPNs) (Bowman & Levine, 2017). Similar to the ESCs from *Tet2*-dificient mice, human cord blood cells depleted for *TET2* and cells isolated from leukemia patients bearing *TET2* mutations exhibit an increase in myeloid lineage differentiation at the expense of the erythroid lineage (Madzo et al., 2014; Pronier et al., 2011). Besides, *TET2* alterations are found in B-cell and T-cell lymphomas. Thus, *TET2* mutations can be involved in very different types of hematological diseases, and recent studies support a role of TET2

mutations as one of the first genetic aberrations in the onset of hematological malignancies (Rasmussen & Helin, 2016).

1.11 TET proteins in non-mammalian systems: flies, frogs and fish

TET orthologs are also present in non-mammalian systems. Interestingly, the Drosophila genome encodes for a single TET ortholog despite having minimal 5mC levels. The function of Drosophila Tet remains undetermined. It is possible that some non-catalytic functions of TET proteins are retained in Drosophila, for instance, recruiting transcriptional partners to target sequences. Alternatively, Drosophila Tet may act on tRNA, as Dnmt2, an RNA methyltransferase, is the only DNMT member identified in this organism (Goll et al., 2006). The ability of Drosophila Tet to rescue *tet* mutant phenotypes in other systems has not been explored.

The Xenopus genome encodes two Tet ortholgs with closest homology to *Tet2* and *Tet3.* Xenopus embryos injected with morpholinos targeting *tet3* exhibit early eye and neural phenotypes (Xu et al., 2012). The phenotype could be partly rescued by injecting mRNA encoding a catalytically inactive TET3, whereas injection with a TET3 CXXC domain mutant failed to rescue the phenotypes. Similarly, homozygous deletion of Tet3 in mice results in eye-related phenotypes including the Eyelid Open at Birth (EOB) phenotype

(Gu et al., 2011). This data suggest an evolutionally conserved function of Tet3 in eye development from vertebrate to mammals.

The zebrafish genome encodes single well-conserved orthologs of *Tet1*, *Tet2* and *Tet3* (Almeida et al., 2012). Previous studies have shown that *tet2* mutant zebrafish exhibit similar MDS phenotypes to those observed in *Tet2* mutant mice (Gjini et al., 2015), suggesting the conserved functions of TET proteins across different species. Intriguingly, while at least *Tet3* is maternally deposited in mouse, few, if any, *tet* transcripts are detected in RNA-seq data from 2-cell stage zebrafish embryos and 5hmC is not detected in the zebrafish embryo by immunofluorescence until the bud stage (Almeida et al., 2012; Gu et al., 2011). Zebrafish do not undergo the same TET dependent erasure and reestablishment of global 5mC patterns observed during mammalian preimplantation development, providing one potential explanation for this distinction (Jiang et al., 2013; Potok et al., 2013). The lack of maternal deposition and limited dependency on TET enzymes during the first 24 hours post fertilization make zebrafish a powerful system for examining TET requirements in later developmental processes, including those associated with tissue specific development and differentiation. A deeper interrogation of TET function in zebrafish is the subject of this thesis.

Section 2. Zebrafish as a model for the study of hematopoiesis

2.1 Overview of hematopoiesis

Blood is among the tissues with the highest cell turnover rate. Billions of new blood cells are generated every day in our bodies to replenish the old ones through hematopoiesis. Hematopoiesis is defined as the development of all types of circulating blood cells derived from single HSC. While normal hematopoiesis needs to be assured to support the life-long requirement of blood cells, abnormal hematopoiesis is associated with a range of blood diseases, including cancer. Complicated genetic and signaling networks are integrated to govern HSC development and orchestrate the balance between self-renewal and differentiation (Orkin & Zon, 2008; Clements & Traver, 2013). A full understanding of HSC development and how HSCs are regulated is a subject of particular interest because of the potential therapeutic applications. For example, de novo expansion of patient-derived HSCs could be used to reconstitute the entire blood system. Despite decades of intense research, generation of fully functional HSCs from pluripotent stem cells at large-scale remains challenging, partly due to the poor understanding of HSC development (Ciau-Uitz, Monteiro, Kirmizitas, & Patient, 2014; Sugimura et al., 2017; Wahlster & Daley, 2016). The internal development of HSCs in mammalian embryos complicates the study of their ontogeny and regulation. Given the surprising degrees of both functional and genetic

conservation between vertebrates, an alternative model to study the development of HSCs and translate human blood diseases is in demand.

2.2 Advantages of the zebrafish model

From studies over two decades, zebrafish has emerged as a powerful system to study developmental and to model human disease. Among its many advantages, the zebrafish model offers large brood size. Each pair of adult zebrafish can produce hundreds of embryos every week, which allows for large-scale screens similar to those performed in flies and worms (Patton & Zon, 2001). Zebrafish embryos also develop rapidly, with gastrulation completed within few hours after fertilization and most of the organs and structures formed within 24 hours post-fertilization (hpf) (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Externally fertilized embryos can be assessed as early as on the one-cell stage, enabling experimentations such as mRNA injection and morpholino knockdown (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009). These transparent embryos are also ideal for the use of fluorescent imaging to study early embryonic processes, including heart development, vascular development, and hematopoiesis.

2.3 Developmental hematopoiesis in zebrafish

Zebrafish provide an excellent platform to study the early development of the hematopoietic system. It is an ideal model for in vivo imaging, and it is useful for large-scale genetic screens. These advantages have led to the discovery of previously unknown players in hematopoiesis, improving our understanding of hematopoietic development.

Most vertebrate experience two waves of hematopoiesis, primitive and definitive hematopoiesis. The transient primitive hematopoiesis gives rise to limited types of blood cells that provide oxygen and innate immunity to the rapidly growing tissues. The definitive wave, which follows the primitive wave, provides all types of blood cells derived from HSC to maintain the blood system throughout life (Figure 3).

Primitive hematopoiesis: Primitive hematopoiesis in zebrafish initiates around 10 hours post fertilization (hpf) and generates mainly macrophages and erythrocytes to support the growth of the early embryos (Jagannathan-Bogdan & Zon, 2013). Two intraembryonic regions have been shown to possess primitive hematopoietic activity: anterior lateral mesoderm (ALM), where most myeloid cells are produced, and posterior lateral mesoderm (PLM), where erythrocytes are produced. Precursor cells at ALM expressing *scl*, *gata2*, *lmo2* and *etsrp* form a structure called rostral blood island (RBI) (Herbomel, Thisse, & Thisse, 1999; Paik & Zon, 2010). These cells then differentiate into macrophages to shape the developing tissues governed by transcription factors like PU.1. Meanwhile, precursor cells at PLM appear as

bilateral stripes and later migrate to central midline and form inner cell mass (ICM) (Detrich et al., 1995). Similar to the mammalian yolk sac island, zebrafish ICM region supports the production and maturation of primitive erythrocytes, which then enter circulation around 24 hpf when the heart starts beating (Figure 3).

Definitive hematopoiesis: While primitive hematopoiesis is initiated by precursor cells that can only give rise to limited types of blood cells, the hallmark of definitive hematopoiesis is the emergence of hematopoietic stem cells (HSCs). These cells can give rise to the full range of erythroid, myeloid and lymphoid lineages to support the life-long requirements for blood cells. Following primitive hematopoiesis, there is a transient wave of definitive hematopoiesis derived from erythromyeloid progenitors (EMP) (Bertrand et al., 2007; McGrath et al., 2011). EMPs are found in posterior blood island (PBI) and have restricted differentiation capability to produce only erythroid and myeloid cells. It has been hypothesized that myeloid cells produced by EMPs can provide innate immune protection to the host and fill the gap before they arise from HSCs (Tober et al., 2007).

At around 30 hpf, HSCs are first detected by the expression of transcription factors *runx1* and *c-myb* in the ventral portion of the dorsal aorta. This region is commonly considered to be the equivalent to the mammalian aorta-gonad-mesonephrons (AGM) region (C. Burns, 2002; Kalev-Zylinska et al., 2002) (Figure 3). Using time-lapse confocal imaging to trace embryos expressing

both endothelial and HSC markers, two seminal studies demonstrated that HSCs directly emerge from transdifferentitaion of endothelial cells underlying dorsal aorta, a process termed endothelial-to-hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa & Herbomel, 2010). During a typical EHT event, elongated endothelial cells start to express HSC markers. These cells gradually change their morphology and bend out into the mesenchyme beneath the dorsal aorta. Subsequently, cells bud off from the posterior cardinal vein and enter the circulation to become functional HSCs. Together with studies in mice (Boisset et al., 2010), these results indicate the endothelial origin of HSCs. The studies also argue for the existence of the hemogenic endothelium, a sub-population of endothelial cells underlying the blood vessels with the capacity to generate HSCs. At 48hpf, when most of the EHT events are completed, newly formed HSCs migrate and seed at posterior tail region called caudal hematopoietic tissue (CHT), where they undergo massive proliferation. By 3 days post-fertilzation (dpf), HSCs migrate to the thymus to initiate lymphogenesis. One day later, HSCs can be detected in the kidney marrow, the ultimate place for adult hematopoiesis. The kidney marrow can be considered analogous to the mammalian bone marrow (H. Jin, Xu, & Wen, 2007; Murayama et al., 2006) (Figure 3).

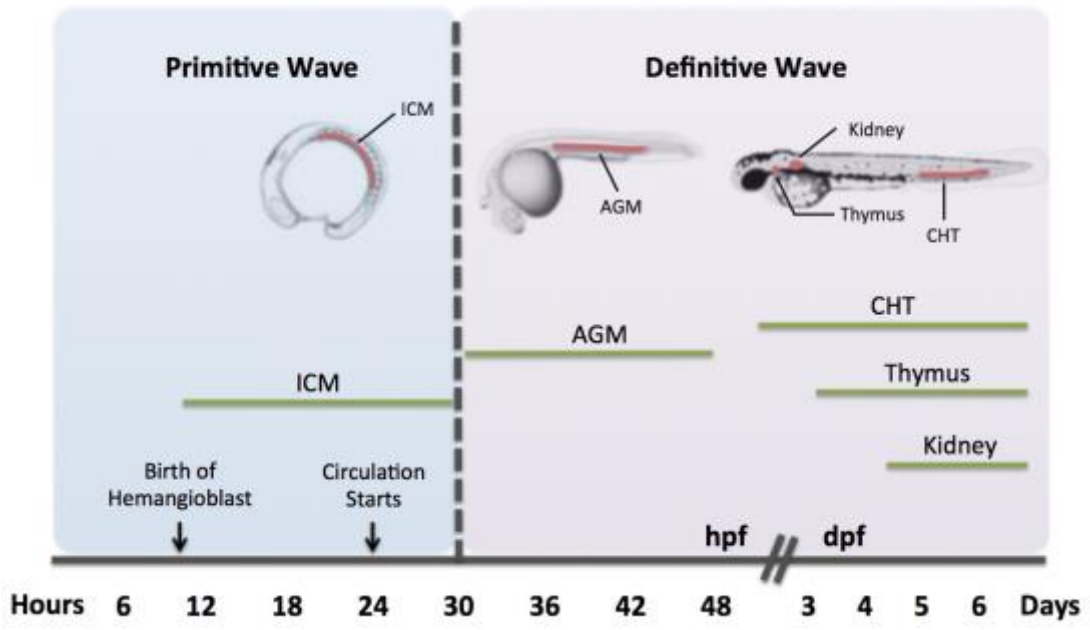


Figure 3. Primitive and definitive hematopoiesis in zebrafish

Primitive erythroid progenitors are born in the intermediate cell mass (ICM). Circulation of primitive erythrocytes starts around 24hpf. The definitive wave begins around 30hpf during which time HSCs emerge from the ventral floor of the dorsal aorta within the aorta-gonad-mesonephros (AGM) region. The HSCs subsequently migrate to the caudal hematopoietic tissue (CHT) in the posterior region of the tail. By 3dpf, the thymus begins lymphopoiesis and HSC migrate to the kidney marrow around 4dpf. Figure based on Jagannathan-Bogdan & Zon, 2013).

2.4 Conservation of regulators of hematopoietic stem cell development between zebrafish and other mammals

Runx1: Runx1 is a transcription factor belongs to the RUNT family. *RUNX1* is a key regulator of hematopoiesis and mutations are often observed in leukemia patients (Gaidzik et al., 2016; Gaidzik et al., 2011). *Runx1* is expressed in functional HSCs in the adult mouse, as well as in cells with

spleen colony-forming unit (CFU) capability (North & de Bruijn, 2004). *Runx1* mutant mice fail to develop definitive blood cells, demonstrating a requirement for Runx1 in definitive hematopoiesis (Okuda, Van Deursen, Hiebert, Grosveld, & Downing, 1996). In zebrafish, *Runx1* is first detected at 5-somite stage in the PLM and later expressed in both neural and hematopoietic tissues. At 30hpf, *runx1* expression can be detected in HSCs that have just emerged from the AGM-like region. Although Runx1 appears to be dispensable for primitive hematopoiesis, it is required for definitive hematopoiesis, as morpholino knockdown of *runx1* results in a loss of *c-myb* expressing cells (Kalev-Zylinska et al., 2002). Similarly, in Runx1-depleted embryos, the hemogenic endothelium fails to maintain its cell integrity and undergoes fragmentation at the onset of EHT (Kissa & Herbomel, 2010). Thus, Runx1 appears to be an important regulator controlling EHT process. Surprisingly, although most *runx1* zebrafish mutants die from lack of definitive hematopoiesis, around 20% mutants survive and are capable of restoring multilineage hematopoiesis (Sood et al., 2010). One explanation for this phenotype is that other members of the Runt family may compensate the loss of Runx1 to reconstitute definitive hematopoiesis.

c-myb: c-myb is a member of the MYB family of proto-oncogenes. *c-myb* is predominantly expressed in immature hematopoietic precursor cells and its expression decreases as these cells differentiate (Paik & Zon, 2010). Depletion of *c-Myb* in mice leads to lethality at gastrulation stage with a failure

in fetal lever erythoropoiesis, suggesting c-Myb is critical for definitive hematopoiesis (Mucenski et al., 1991). In zebrafish, *c-myb* expression is detected in *runx1*-expressing cells in the AGM-like region starting from 30 hpf. As a faithful HSC marker, *c-myb* expression can also be detected in the CHT at 2 dpf and later in thymus and the kidney (H. Jin et al., 2007; Murayama et al., 2006). Similar to Runx1, c-myb is dispensable for primitive hematopoiesis but is required for the definitive hematopoiesis, as *c-myb* mutant fish have complete loss of definitive blood cells (Soza-Ried, Hess, Netuschil, Schorpp, & Boehm, 2010). It has been demonstrated that c-myb facilitates the migration of HSCs from the ventral wall of dorsal aorta to CHT, which explains the failure of initiating definitive hematopoiesis in *c-myb* mutant embryos (Zhang, Jin, Li, Qin, & Wen, 2011).

Scl: Scl encodes a basic helix-loop-helix (bHLH) transcription factor. In the mouse embryo, *Scl* is expressed in hemogenic sites including yolk sac blood islands, fetal liver, and the dorsal aorta (Paik & Zon, 2010). Additionally, *Scl* mutant mice are not able to initiate primitive hematopoiesis, suggesting its requirement in the primitive wave (Shivdasani, Mayer, & Orkin, 1995). During zebrafish development, *scl* is expressed around 2-somite stage in hemangioblast population together with *gata2*, *lmo2* and *etsrp*. Scl is a key regulator of hemagioblast as morpholino knockdown of *scl* leads to severe defects in both vasculogenesis as well as primitive hematopoiesis (Dooley, Davidson, & Zon, 2005; Patterson, Gering, & Patient, 2005; Ren, Gomez,

Zhang, & Lin, 2010). Scl has also been shown to regulate definitive hematopoiesis. In zebrafish, two *scl* isoforms, ***sclα*** and ***sclβ***, regulate HSC development at different stages (Qian et al., 2007; Zhen, Lan, Yan, Zhang, & Wen, 2013). ***Sclβ*** is expressed in the ventral wall of dorsal aorta and is required to specify the hemogenic endothelium, as Sclβ-depleted embryos have elevated numbers of apoptotic endothelial cells prior to the onset of EHT. In contrast, the ***sclα*** expression starts to accumulate after HSCs have budded off from the AGM-like region. Morpholino knockdown of ***sclα*** results in intact hemogenic endothelium specification but a loss of HSC population after EHT, demonstrating the specific requirements for different *scl* isoforms during HSC development.

Gata2b: Gata2 is a well-known zinc finger transcription factor co-expressed with *scl* in hemangioblast at 2-somite stage. In mice, Gata2 is regulated by Notch signaling and is required within the endothelium for the expression of *runx1* (Robert-Moreno, Espinosa, de la Pompa, & Bigas, 2005). Zebrafish genome encodes two *gata2* paralogs, *gata2a* and *gata2b*. Morpholino knockdown of *gata2a* in zebrafish results in mild defects in primitive hematopoiesis (Galloway, Wingert, Thisse, Thisse, & Zon, 2005), nevertheless, *gata2a* mutant embryos display defects in aorta morphogenesis and circulation, indicating its primary role in regulating vasculogenesis (C. Zhu et al., 2011). A recent study reveals *gata2b* is specifically expressed in the hemogenic endothelium prior to the expression of *runx1* (Butko et al.,

2015). Additionally, *gata2b*-exprssing cells are capable of giving rise to adult hematopoietic cells, while embryos depleted of *gata2b* lose embryonic HSCs. Taken together, these data suggest that genome duplication in zebrafish endows subspecialized functions to Gata2b, which primarily controls HSC development by specifying the hemogenic endothelium.

Notch Signaling: Notch signaling pathway controls cell fate and pattern formation. Studies in mice have demonstrated that defective Notch signaling pathway leads to decreased number of HSCs. Conversely, activation of Notch signaling pathway expands HSC population (Guruharsha, Kankel, & Artavanis-Tsakonas, 2012; Robert-Moreno et al., 2005, 2008). Zebrafish *mindbomb* mutant, which was first found in a forward genetic screen and studied for defective neural development, has defective Notch signaling activity due to the mutation in ubiquitin E3 gene required for Notch ligand processing (Itoh et al., 2003). Despite having intact primitive hematopoiesis and vasculogenesis, *mindbomb* mutants fail to specify HSCs (C. E. Burns, Traver, Mayhall, Shepard, & Zon, 2005). Transient expression of Notch intracellular domain rescues *runx1*+ *c-myb*+ cells. Subsequent studies have demonstrated that both cell autonomous and non-cell autonomous Notch signaling are required for the regulation of HSC development (Albert D Kim et al., 2014). *Notch1a* and *Notch1b* are expressed within the hemogenic endothelium and morpholino knockdown of either of these two Notch receptors reduces the number of HSCs. Importantly, the defect can be

rescued by activating Notch signaling at 20 hpf, the time when HSC precursors first experience Notch signaling. *Notch3*, on the other hand, is expressed in somite at 13 hpf. Morpholino knockdown of *Notch3* in the embryos leads to decreased HSC population which can only be rescued by induction of Notch signaling at 14 hpf, indicating Notch3 is required non-cell autonomously. These results highlight the importance of Notch signaling during HSC development.

BMP Signaling: Bone morphogenic proteins are a group of growth factors regulating tissue patterning and architecture. In mammals, chemical inhibition of BMP signaling leads to reduced HSC numbers contained within the AGM region (Durand et al., 2007). In addition, mouse AGM explants that contain nascent HSCs exhibit enhanced repopulation potential with the addition of Bmp4. These studies highlight the importance of BMP signaling in regulating HSC development. During zebrafish development, a reciprocal hedgehog-BMP gradient appears to be critical to polarize HSC formation from the ventral wall of the dorsal aorta (Wilkinson et al., 2009). Bmp4-inhibited embryos have intact arterial programs but fail to maintain the *runx1* expression and lack HSC population. It has been shown that BMP antagonists, such as *noggin* and *gremlin*, are expressed in tissues surrounding dorsal aorta prior to the appearance of HSC precursors. This Bmp4 repressive environment is at least partially regulated by FGF signaling (Pouget et al., 2014). Right before the onset of *runx1* expression, expression

of these BMP antagonists reduces and *bmp4* starts to express, switching Bmp4 repressive environment to BMP4 supportive environment to facilitate HSC emergence (Wilkinson et al., 2009).

Wnt Signaling: Wnt signaling is crucial for stem cell development and function. Although most studies have confirmed a positive link between canonical Wnt signaling and HSC development (Reya et al., 2003; Willert et al., 2003), some studies suggest otherwise (Kirstetter, Anderson, Porse, Jacobsen, & Nerlov, 2006; Scheller et al., 2006). It is likely that Wnt signaling regulates HSC development in a dosage-dependent manner (Tiago C. Luis et al., 2011, 2012). A chemical screen performed in zebrafish in search of molecules that alter the size of HSC population reveals PGE2 as a positive regulator of HSC development (Trista E North et al., 2007). Later studies demonstrate PGE2 acts via cAMP/PKA to regulate Wnt-mediated HSC development (Goessling et al., 2009). Studies in zebrafish also reveal an important role of non-canonical Wnt signaling through Wnt16 ligand in regulating HSC development (Clements et al., 2011). Wnt16, together with FGF signaling and junction adhesion molecules, indirectly regulates HSC development by controlling the expression of two Notch ligands *deltaC* and *deltaD* in the somite (Clements et al., 2011; Kobayashi et al., 2014; Lee et al., 2014). Morpholino knockdown of *wnt16* abolishes *runx1*+ and *c-myb*+ cells with intact vasculature and arterial specification. Transient expression of Notch signaling at 14 hpf rescues *runx1*+ cells at dorsal aorta. It has been shown that Notch receptor Notch3

orchestrates the non-canonical wnt16 signaling pathway and regulates HSC development non-cell autonomously (Albert D Kim et al., 2014).

Inflammatory Signaling: Inflammatory signaling pathways are composed of secreted proteins and cytokines responsible for cell stress and infection. Their roles in regulating normal development are just beginning to emerge, and recent findings have unmasked the surprising involvements of inflammatory signaling during HSC development. Inflammatory signaling plays an evolutionarily conserved role in promoting definitive hematopoiesis from fish to mammals (He et al., 2015). In zebrafish, inflammatory signaling is highly enriched in hemogenic endothelium. IFNγ has been described to positively regulate HSC emergence in a cell autonomous manner downstream of Notch signaling (Sawamiphak, Kontarakis, & Stainier, 2014). Another inflammatory cytokine, TNFα, stimulates the expression of Notch ligand *Jagged1a* in endothelial cells and activates Notch signaling in the nearby hemogenic endothelium through Notch1 receptor (Espín-Palazón et al., 2014), a pathway that has been shown to be conserved between zebrafish and mammals (He et al., 2015; Li et al.,2014). Interestingly, these cytokines are secreted from primitive neutrophils and thus act in a non-cell autonomous manner to regulate HSC development (Espín-Palazón et al., 2014; He et al., 2015).

2.5 Epigenetic regulation of hematopoiesis: early lessons from the zebrafish model

Studies in mice, cell culture and zebrafish have identified a number of transcription factors and signaling pathways that are important for HSC development (Jagannathan-Bogdan & Zon, 2013). However, much less is known about how epigenetic regulation contributes to the production and maintenance of HSCs in the developing embryo. The accessibility of early stages of HSC development and large brood sizes makes zebrafish well suited to genetic approaches aimed at identifying epigenetic regulators of HSC development. The application of forward and reverse genetic approaches in zebrafish is beginning to unravel the relevant epigenetic pathways.

For example, a recent candidate screen for chromatin factors that regulate zebrafish hematopoiesis substantially expanded the number of epigenetic regulators implicated in HSC development. Screening of a panel of 425 morpholinos that depleted proteins containing amino acid motifs associated with chromatin or nucleic acid binding revealed 31 candidates that caused strong alterations in *runx1*/*c-myb* expression levels (Huang et al., 2013). Among these candidates, 20 morpholinos impacted HSC development without negative effects on vascular or arterial development. Consistent with previous studies, this candidate screen implicated enzymes involved in histone modification including *hdac1* and components of the PRC1 and SET1/trithorax complexes. In addition, several chromatin modifiers that had not been previously implicated in HSC development were uncovered,

including *brd8a, jmjd1* and *nap1l4a*. An added advantage of the large-scale candidate approach is that it allows for identification of multi subunit complexes that include more than one gene implicated by the screen. This study demonstrates that candidate screen approach can be used to further evaluate epigenetic components in HSC development.

A more extensive review of epigenetic regulation of hematopoiesis in zebrafish can be found in Appendix II.

CHAPTER TWO: EXPERIMENTAL RESULTS

Section 1. Overlapping requirements for Tet2 and Tet3 in normal zebrafish development

1.1 Systemic analysis of Tet gene requirements during zebrafish development

The zebrafish genome encodes single well-conserved orthologs of *TET1*, *TET2* and *TET3* (Almeida et al., 2012). To systematically define requirements for these genes during development, we introduced mutations into the zebrafish *TET* ortholgs using TAL effector nucleases (TALENs) (Li et al., 2011a; Sander et al., 2011). RNAs encoding TALENs that targeted *tet1*, *tet2* and *tet3* were separately injected into one-cell stage embryos and individuals harboring germline transmissible mutations in each of the three genes were recovered. The recovered *tet2*^{mk17} allele deletes 4 base pairs in exon 8. This deletion results in a frame shift, causing early termination one amino acid 3' of an essential iron-binding residue (Figure 4A and 4B). The *tet1*^{mk16} and *tet3*^{mk18} alleles harbor 4 and 14 base pair deletions, respectively, in the last coding exon. In addition to causing frame shift and premature termination, these deletions eliminate sequence encoding a C-terminal arginine residue that is required for 2-oxoglutarate binding (Hu et al., 2013) (Figure 4A and 4B). Loss of specific residues involved in cofactor binding and catalysis is predicted to similarly compromise the dioxygenase activity of all three

enzymes. Zebrafish lines were then intercrossed to generate larvae that were homozygous mutant for all single, double and triple combinations.

1.2 Tet2 and Tet3 are the major 5mC dioxigenases in the zebrafish embryo

To assess 5hmC levels in the mutants, we performed dot blots using an antibody that is specific for the 5hmC modification. Modest (less than 3 fold) reductions in total 5hmC were observed in larvae that were homozygous mutant for *tet1*, *tet2*, or *tet3* (Figure 4C). More considerable reductions in 5hmC were observed in double mutants, with the *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/mk18}$ double mutant (*tet2/3*^{DM}) combination producing the most dramatic decrease (>30-fold) (Figure 4C). 5hmC levels were further reduced in *tet1/2/3* triple mutant (*tet1/2/3*^{TM}) larvae; indicating that all three mutated genes encode proteins that are compromised for catalytic activity (Figure 4D). The enhanced loss of 5hmC in *tet2/3*^{DM} larvae compared to other double mutant combinations argues that Tet2 and Tet3 are the predominant 5mC oxidases in the zebrafish embryo and that they function redundantly to promote the formation of 5hmC during development.

1.3 Tet requirements during zebrafish development

Zebrafish that were homozygous for mutations in *tet1*, *tet2* or *tet3* were viable to adulthood and fertile, as were *tet1/2* and *tet1/3* double homozygous mutants. In contrast, combined mutation of *tet2* and *tet3* was not compatible with survival beyond the larval period. The *tet2/3*^{DM} larvae were

morphologically indistinguishable from wild-type controls during the first 24 hours post fertilization (hpf), but subtle abnormalities in brain development emerged on the second day post fertilization (dpf). By 36 hpf, smaller eyes, abnormal brain morphology, altered pigmentation and a modest curvature of the trunk were apparent in 25% of larvae derived from intercrosses between *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ adults (Figure 4E). Genotyping (n=20) confirmed that the morphologically abnormal embryos all carried homozygous mutations in both *tet2* and *tet3,* demonstrating a combined requirement for the two genes in zebrafish development. Despite the additional reduction in 5hmC, *tet1/2/3*^{TM} larvae were morphologically indistinguishable from *tet2/3* double mutants at all stages examined (Fig 4F).

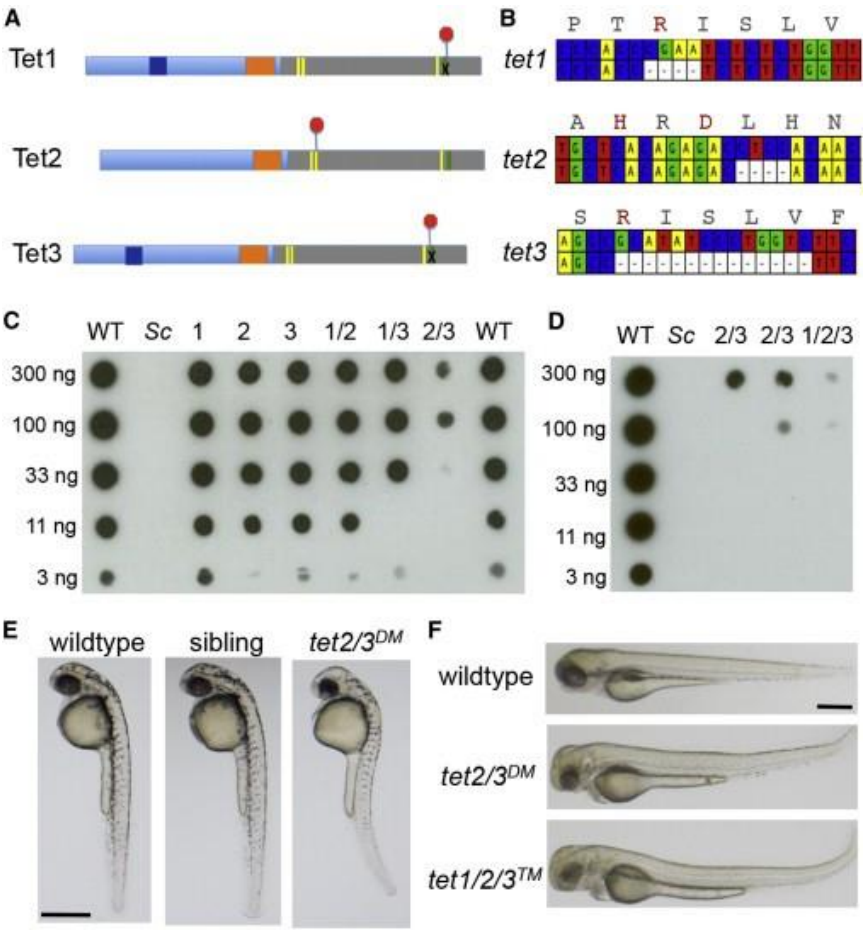


Figure 4. Mutation of zebrafish *tet1*, *tet2*, and *tet3*

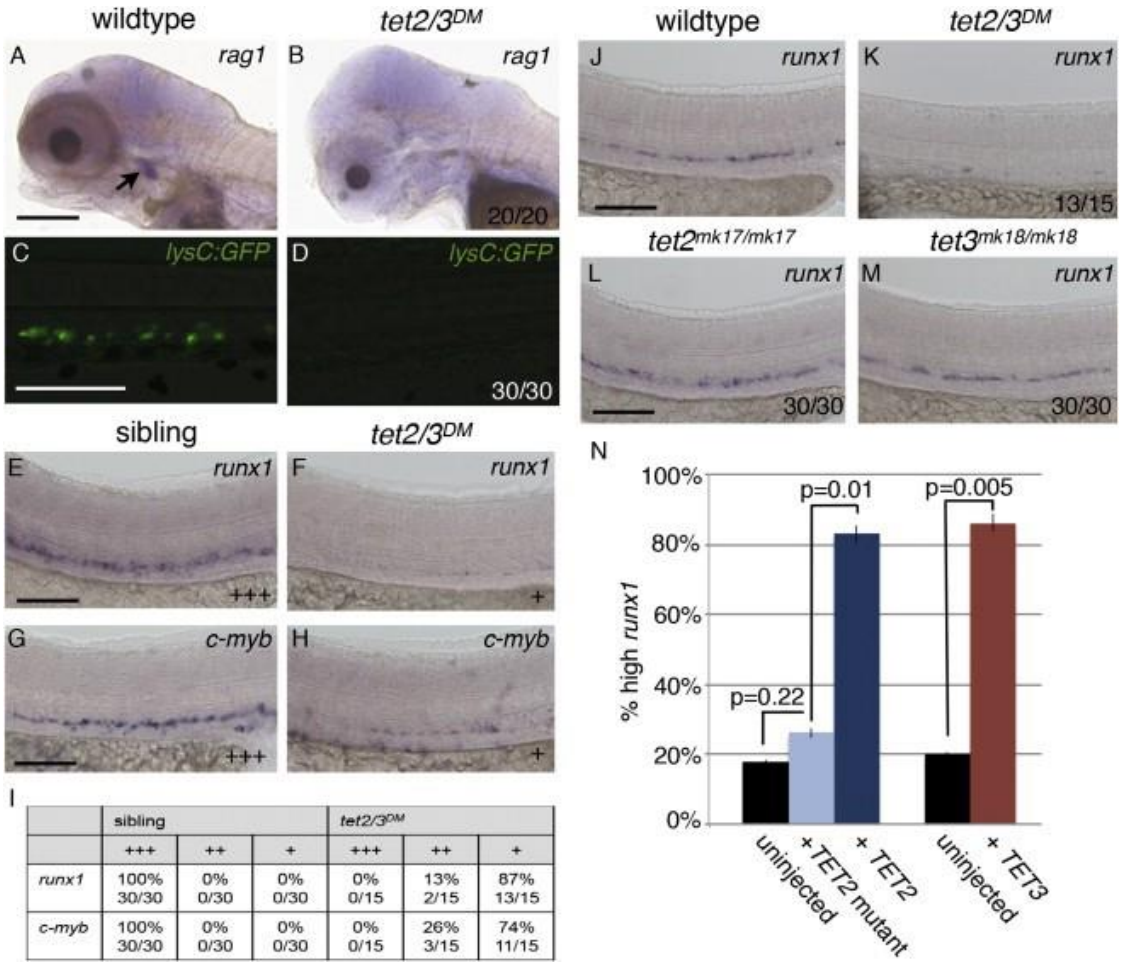
(A) Schematic illustrating early termination caused by TALEN mutations in zebrafish *tet1*, *tet2* and *tet3*. Red octagons indicate the position of early termination signals. Yellow bars indicate conserved iron binding residues, the green bar indicates the arginine required for 2-oxogluterate binding. (B) Schematic depicting deleted bases in zebrafish *tet1*, *tet2* and *tet3*. Corresponding amino acid sequences for the wild- type allele are included, with residues required for cofactor binding or catalysis indicated in red. (C) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf. Numbers indicate the mutated *tet* gene(s) in each sample. *Sc* indicates DNA isolated from *Saccharomyces cerevisiae.* Horizontal rows depict 3-fold serial dilutions of DNA. (D) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf including DNA isolated from *tet1/2/3*^{TM} larvae. (E) Lateral views of a representative WT larva, a *tet2/3*^{DM} larva and a sibling larva derived from a *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercross at 36 hpf. (F) Lateral views of a representative WT larva, a *tet2/3*^{DM} larva and *tet1/2/3*^{TM} larva at 3 dpf.

Section 2. Overlapping requirements for Tet2 and Tet3 in HSC emergence

2.1 Mutation of tet2 and tet3 causes loss of definitive blood

Previous studies have suggested that TET regulation is of particular importance in hematopoietic lineage, and many studies have shown the correlation of *TET* mutations with various types of myeloid malignancies. While the importance of TET regulation in the adult hematopoietic system is clear, less is known about requirements for *Tet* genes during early stages of hematopoietic development. Given the combinatorial effects of *tet2* and *tet3* mutation on overall development, and the known roles of TET proteins in later stages of hematopoiesis, we tested whether *tet2* and *tet3* had overlapping roles in the development of the definitive hematopoietic system.

Consistent with a role for TETs in the early development of the hematopoietic system, our initial analysis revealed that *tet2/3*^{DM} larvae lacked differentiated blood cells associated with the definitive wave. For example, while mature *rag1*-expressing T-cells were readily observed in the thymus of wild-type larvae at 5 dpf, *rag1* positive cells were absent from the corresponding region of *tet2/3*^{DM} larvae (Figure 5A-5B). Similarly, the *Tg*(*lysC:GFP)* transgenic line, which labels definitive granulocytes (Hall et al., 2007), produced a clear fluorescent signal in blood from wild-type larvae at 6 dpf, but fluorescent cells were not detected in *tet2/3*^{DM} larvae at this stage (Figure 5C-5D).



I

	sibling			tet2/3DM		
	+++	++	+	+++	++	+
runx1	100% 30/30	0% 0/30	0% 0/30	0% 0/15	13% 2/15	87% 13/15
c-myb	100% 30/30	0% 0/30	0% 0/30	0% 0/15	26% 3/15	74% 11/15

Figure 5. *tet2* and *tet3* have overlapping functions in the HSC development

(A and B) WISH for *rag1* at 5 dpf. Arrow indicates thymic T-cells in wild-type larvae. (C and D) GFP labeled macrophages and neutrophils in 6 dpf larvae carrying the *Tg(lysC:GFP)* transgene. (E and F) WISH for the HSC marker *runx1* in the DA at 36 hpf. (G and H) WISH for the HSC marker *c-myb* in the DA at 36 hpf. (I) Number of sibling and *tet2/3*^{DM} embryos with wild type (+++), reduced (++), or nearly absent (+) *runx1* labeling in the DA. Numbers are representative of three independent crosses. (J-M) WISH for *runx1* in the DA at 32 hpf. (N) Graph indicating the percent of *tet2/3*^{DM} larvae exhibiting high *runx1* staining in the DA in uninjected controls or following injection with 100 pg of mRNA encoding TET2, TET2 H1382Y.D1384A (TET2 mutant) or TET3. Numerical data is presented as the mean ± SEM.

2.2 Hematopoietic stem cell markers are not detected in tet2/3^{DM} larvae

The lack of differentiated blood cells derived from HSCs led us to next address whether HSC development was impaired in double mutants. During normal zebrafish development, HSCs emerge from the hemogenic endothelium in the ventral wall of the dorsal aorta (DA) starting from 30 hpf. In *tet2/3^{DM}* larvae, we found the expression of the HSC-associated genes, *runx1* and c-*myb* was reduced in the DA at these stages, whereas the *runx1* expression in *tet2* and *tet3* single homozygous mutant larvae appeared indistinguishable from wildtype (Figure 5E-5M**)**. Moreover, in *tet2/3^{DM}* larvae, *c-myb* positive hematopoietic stem and progenitor cells (HSPCs) could not be detected in the caudal hematopoietic tail (CHT) niche, a secondary, transient site for HSC amplification (Figure 6A-6F). All together, these observations suggest the HSC development is impaired in the double mutants.

To confirm the specificity of the HSC phenotype observed in the double mutant larvae, we tested whether the phenotype could be rescued by reintroducing mRNA encoding different TET proteins. We found that injecting mRNA encoding either human TET2 or TET3 into one-cell stage embryos derived from *tet2$^{mk17/mk17}$*, *tet3$^{mk18/+}$* intercrosses rescued the *runx1* expression, whereas injection of mRNA encoding a catalytically dead version of TET2 did not (Figure 5N). In addition to confirming the specificity of the HSC phenotype in *tet2/3^{DM}* larvae, the rescue experiment directly implicates

the importance of 5mC dioxygenase activity of TET2 in regulating HSC development (Figure 5N).

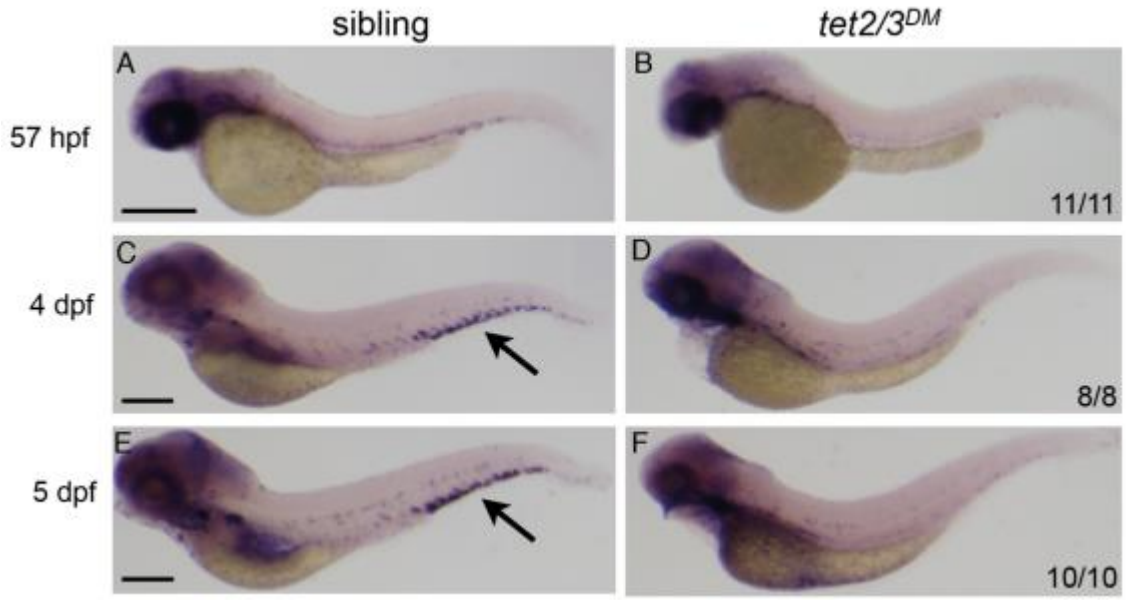


Figure 6. Reduced labeling of *c-myb* positive hematopoietic stem and progenitor cells in *tet2/3*^{DM} larvae (A-B) WISH for *c-myb* at 57 hpf. (C-D) WISH for *c-myb* at 4 dpf. Arrow indicates the caudal hematopoietic tissue (CHT). (E-F) WISH for *c-myb* at 5 dpf. Arrow indicates the caudal hematopoietic tissue (CHT).

2.3 Vascular development and blood flow are overtly normal in tet2/3^{DM} *larvae*

Normal vascular development, arterial specification, and blood flow-induced Nitric Oxide (NO) signaling are known prerequisites for HSC development (Adamo et al., 2009; Jagannathan-Bogdan and Zon, 2013; North et al., 2009). Therefore we next investigated whether defects in vascular development or blood flow might underlie HSC defects in *tet2/3*^{DM} larvae. WISH for the vascular markers *kdrl* and *cdh5,* and the arterial marker *efnb2a* revealed

similar expression in wildtype and *tet2/3*^{DM} larvae, demonstrating the presence of an overtly intact vasculature (Figure 7A-7F). Similarly, visual inspection of *tet2/3*^{DM} larvae by bright field microscopy showed that blood flow was grossly normal during the first two days of development, and *klf2a*, an immediate early responder to blood flow, was expressed at similar levels in wild-type and *tet2/3*^{DM} larvae (Figure 7G-7H).

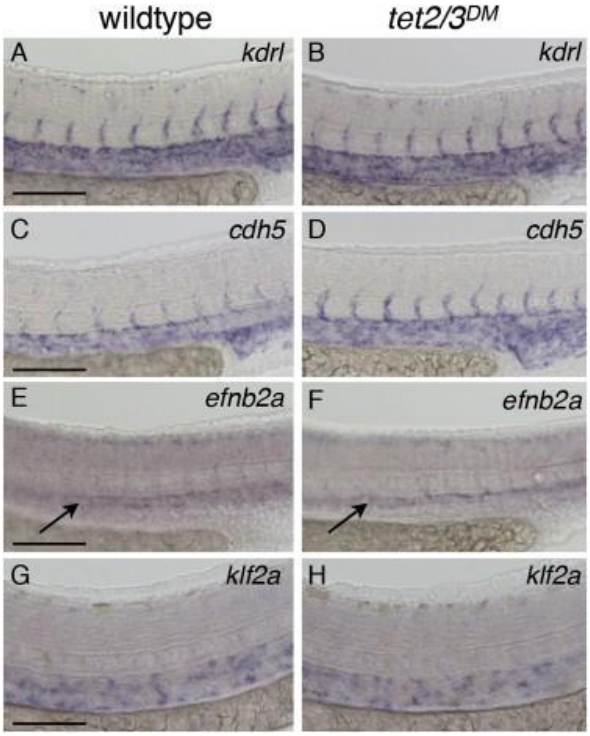
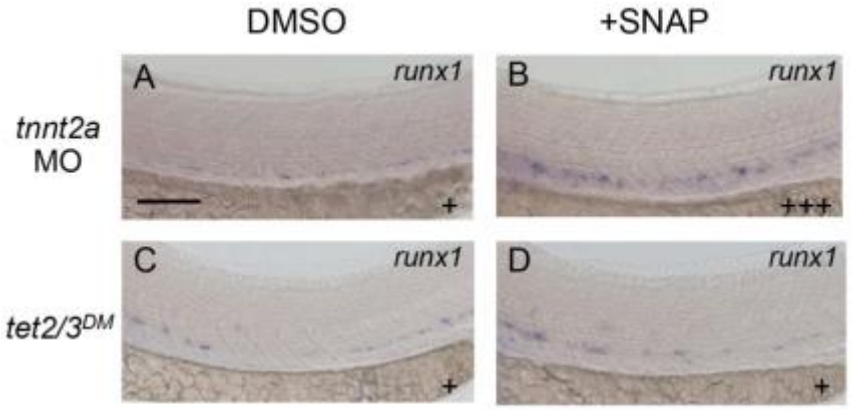


Figure 7. Vascular development and blood flow are overtly normal in *tet2/3*^{DM} larvae.
(A and B) WISH for the vascular marker *kdrl at* 31 hpf. (C and D) WISH for vascular marker *cdh5* at 31 hpf. (E and F) WISH for the arterial marker *efnb2a* at 31 hpf. (G and H) WISH for the blood flow dependent marker *klf2a* at 36 hpf.

In addition, while exposure to the NO agonist S-nitroso-N-acetyl-penicillamine (SNAP) was sufficient to rescue *runx1* expression in *silent heart* (*sih*) morpholino injected embryos lacking blood circulation, SNAP exposure was

unable to rescue *runx1* expression in *tet2/3*^{DM} larvae (Figure 8A-8E). Taken together, these results suggest that the HSC defects observed in *tet2/3*^{DM} larvae are not secondary to defects in vascular development or aberrant blood flow.



E

	DMSO			SNAP		
	+++	++	+	+++	++	+
WT	97% 34/35	3% 1/35	0% 0/35	91% 33/36	8% 3/36	0% 0/36
tnnt2a MO	0% 0/37	54% 20/37	46% 17/37	39% 11/28	54% 15/28	7% 2/28
tet2/3^{DM}	13% 3/23	34% 8/23	52% 12/23	9% 2/21	24% 5/21	66% 14/21

Figure 8. SNAP does not rescue HSC development in *tet2/3*^{DM} larvae.

(A-B) WISH for *runx1* in the DA of 36 hpf *tnnt2a* morpholino-injected embryos following exposure to DMSO or SNAP. (C-D) WISH for *runx1* in the DA of 36 hpf *tet2/3*^{DM} embryos following exposure to DMSO or SNAP. (E) Number of *tet2/3*^{DM} larvae exhibiting high (+++), low (++) or negligible (+) *runx1* expression in the DA at 36 hpf following exposure to DMSO or SNAP.

2.4 tet2/3^{DM} *larvae exhibit impaired hematopoietic stem cell emergence*

Beginning around 32 hpf, nascent HSCs emerge from the ventral aortic endothelium of the zebrafish embryo through a process termed the

endothelial to hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa and Herbomel, 2010). To test whether this process was impacted by mutations of *tet2* and *tet3*, the *tet2* and *tet3* mutant alleles were introduced into a *Tg(kdrl:Ras-mCherry)*^{S896}, *Tg(kdrl:H2B-EGFP)*^{mu122} transgenic background (Chi et al., 2008; Kochhan et al., 2013). In this background, membrane mCherry and nuclear GFP are expressed in the vascular endothelium and emergent HSCs, allowing EHT events to be identified based on stereotypical changes in cell morphology (Bertrand et al., 2010; Kissa and Herbomel, 2010). Prior to the onset of EHT, *tet2/3*^{DM} larvae and siblings from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses exhibited similar fluorescent labeling with the two transgenes (Figure 9A-9B). A defined region of the DA was then monitored in *tet2/3*^{DM} larvae and sibling controls (n=3 each) between 30 and 46 hpf by time-lapse confocal microscopy. Analysis of image sets revealed a four-fold reduction in the number of EHT events detected in *tet2/3*^{DM} larvae compared to siblings, indicating that EHT is compromised in the double mutants (average of 3 vs 13 EHT events, p=0.001; Figure 9C and 9E).

In each time-lapse sequence from *tet2/3*^{DM} larvae, we also observed between one and three cells within the DA undergoing nuclear fragmentation (Figure 9D and 9F). In contrast, fragmentation was never detected in time-lapse sequences from siblings (Figure 9D). The nuclear fragmentation phenotype we observed in *tet2/3*^{DM} larvae is reminiscent of that described for zebrafish embryos depleted for *runx1* by morpholino injection, and suggests that a

fraction of cells undergoing EHT also die via apoptosis in the absence of *tet2/3* (Kissa and Herbomel, 2010).

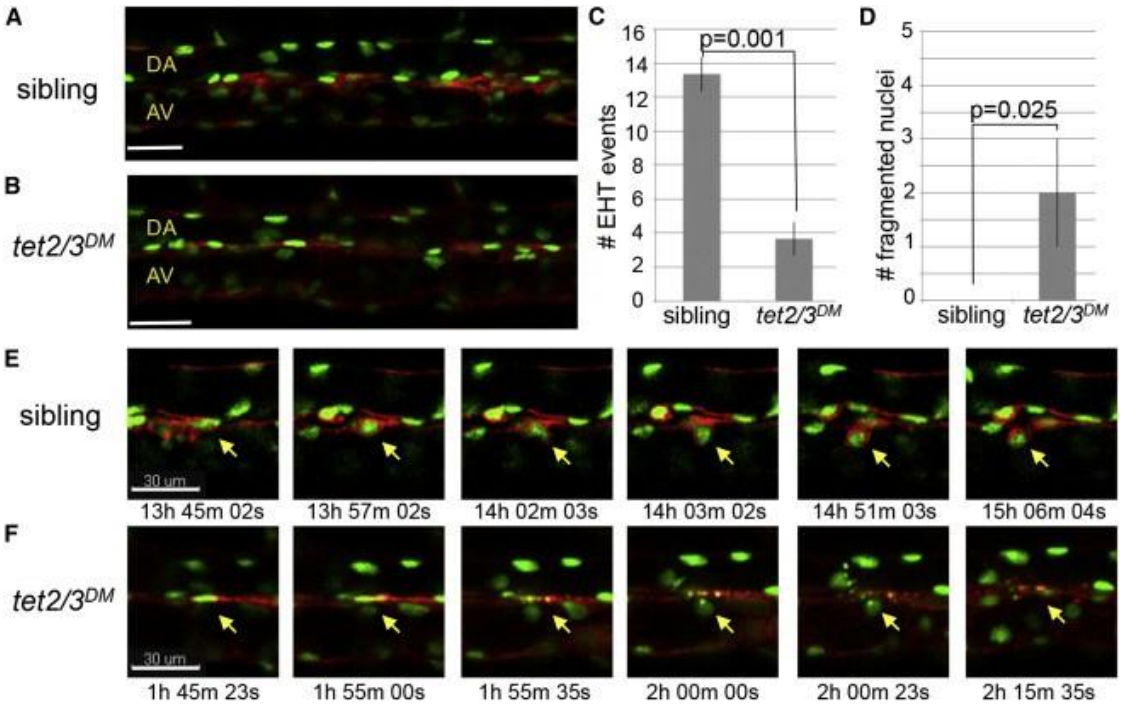


Figure 9. *tet2* and *tet3* are required for the emergence of HSC through the endothelial to hematopoietic transition

(A and B) Merged images depicting GFP and mCherry labeling of the vasculature in *Tg(kdrl:Ras-mCherry)*, *Tg(kdrl:H2B-EGFP)* transgenic larvae at 30 hpf. (C) Number of EHT events detected between 30 and 46 hpf in *tet2/3*^{DM} larvae and siblings. Numerical data is presented as the mean ± SEM. (D) Number of fragmented nuclei observed in the DA of *tet2/3*^{DM} larvae and their siblings between 30 and 46 hpf. Numerical data is presented as the mean ± SEM. (E) Sequences documenting the stepwise emergence of an HSC from the DA of a sibling larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell undergoing EHT. (F) Sequences documenting a cell undergoing nuclear fragmentation in the DA of a *tet2/3*^{DM} larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell with nuclear fragmentation.

2.5 Tet2 and Tet3 regulated hematopoietic transcription factors in the hemogenic endothelium

The reduction in EHT events in *tet2/3*^{DM} larvae suggested a requirement for Tet2/3 in the function or specification of the hemogenic endothelium, which gives rise to nascent HSCs. The hematopoietic transcription factors *runx1* and *scl* are both expressed in the hemogenic endothelium prior to the initiation of EHT and are required for this process (Kissa and Herbomel, 2010; Zhen et al., 2013). By WISH, we found that expression of both *runx1* and *scl* was reduced in the DA of *tet2/3*^{DM} larvae prior to HSC emergence, suggesting that Tet2 and Tet3 are required to promote the hemogenic potential of the vascular endothelium (Figure 10A-10D). Expression of *scl* and *runx1* is controlled in part by the transcription factor Gata2 in mouse, and the zebrafish genome encodes for two Gata2 paralogs that have undergone subfunctionalization (Butko et al., 2015; Gao et al., 2013; Gottgens et al., 2002; Pimanda et al., 2007). Zebrafish *gata2a* is broadly expressed throughout the hematopoietic system, and is important for vascular morphogenesis (Zhu et al., 2011). In contrast, zebrafish *gata2b* is specifically detected in the hemogenic endothelium and is required for *runx1* expression within this tissue (Butko et al., 2015). Consistent with the HSC specific phenotypes observed in *tet2/3*^{DM} larvae, we found that *tet2/3* mutation compromised expression of *gata2b*, while *gata2a* expression was unaffected at similar stages (Figure 10E-10H). Collectively, these observations identify

Tet2/3 as essential regulators of the *gata2b/scl/runx1* transcriptional network in the hemogenic endothelium.

To clarify whether the disruption of *gata2b/scl/runx1* transcriptional program could account for the HSC defects observed in *tet2/3*^{DM} larvae, we next tested whether reintroducing mRNA encoding *scl* or *gata2b* into *tet2/3*^{DM} embryos could rescue HSC production. *In vitro* transcribed mRNA encoding *scl* or *gata2b* was injected into one cell-stage embryos derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses and *runx1* expression was subsequently examined in the DA of *tet2/3* double mutants by WISH at 36 hpf. Injection of either mRNA was sufficient to rescue wild-type levels of *runx1* expression in the DA of *tet2/3*^{DM} larvae, identifying the *gata2b/scl/runx1* network as the primary hematopoietic program regulated by Tet2/3 during HSC emergence (*scl*: p=0.0004, *gata2b*: p=0.0008, Figure 10L-10M).

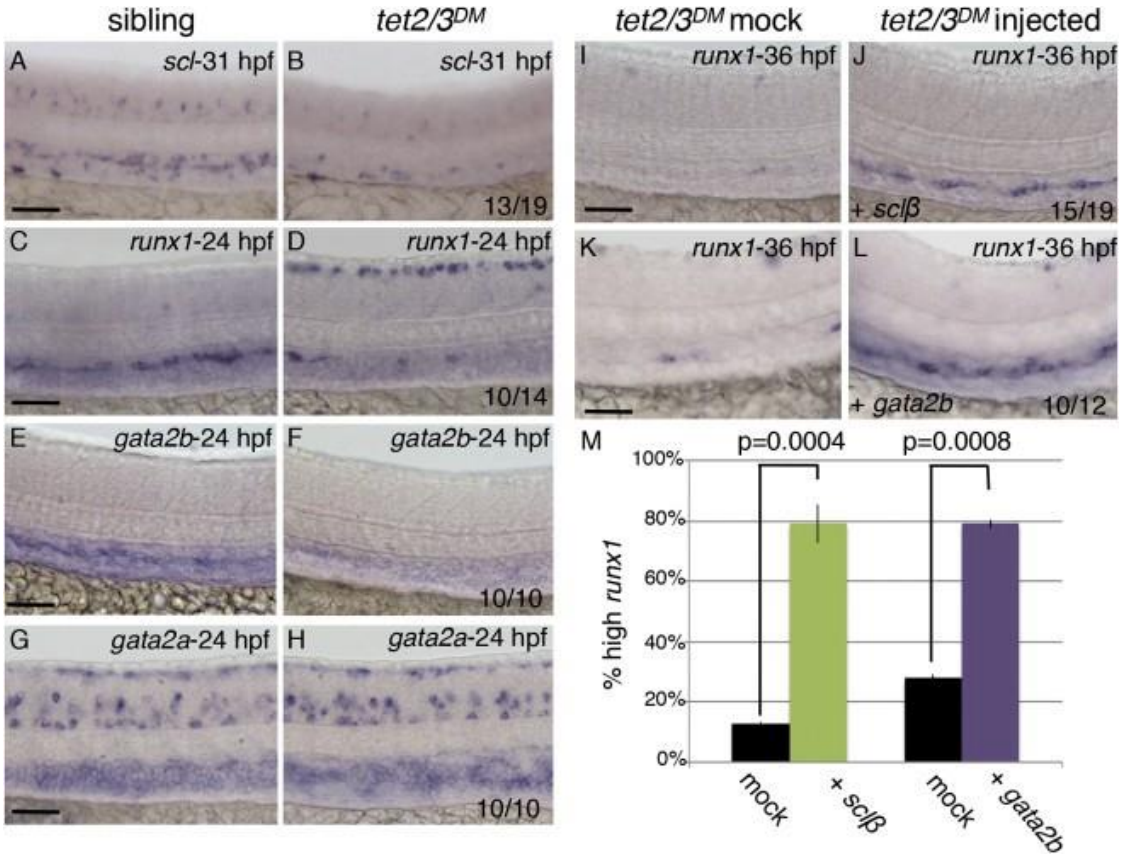


Figure 10. Tet2/3 regulate expression of the *gata2b/scl/runx1* transcriptional network in the hemogenic endothelium

(A-B) WISH for *scl* at 31 hpf. (C-D) WISH for *runx1* at 24 hpf. (E-F) WISH for *gata2b* at 24 hpf. (G-H) WISH for *gata2a* at 24 hpf. (I-J) WISH for *runx1* in the DA of uninjected and ***sclβ*** mRNA-injected *tet2/3*^{DM} embryos at 36 hpf. (K-L) WISH for *runx1* in the DA of uninjected and *gata2b* mRNA-injected sibling embryos at 36 hpf. (M) Percent of *tet2/3*^{DM} embryos with high *runx1* expression following mock injection, injection with mRNA encoding Scl*β* (50pg) or Gata2b (200pg). Numerical data is presented as the mean ± SEM.

2.6 Tet2 and 3 are required for Notch signaling in the hemogenic endothelium

Analysis of genomic DNA from sorted vascular endothelial cells revealed that the promoters of *gata2b*, *runx1* and *scl* were similarly unmethylated in wildtype and *tet2/3*^{DM} larvae, suggesting that Tet2/3 do not directly regulate expression of these genes via promoter demethylation (Figure 11). This observation raised the possibility that Tet2/3 act upstream of *gata2b* in regulating HSC emergence. Notch signaling is required for the expression of *gata2b* in the zebrafish hemogenic endothelium and Gata2 is a direct target of Notch signaling in the mouse dorsal aorta, making this pathway a strong candidate for Tet2/3 regulation during HSC emergence (Butko et al., 2015; Robert-Moreno et al., 2005).

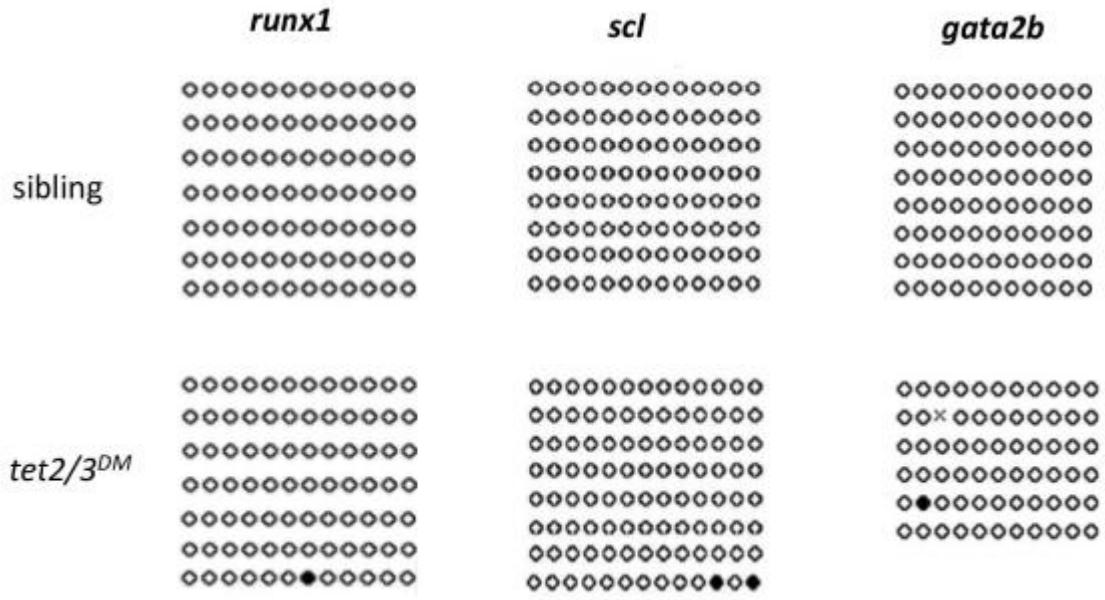


Figure 11. Promoter methylation at *runx1*, *scl* and *gata2b* is unaffected in *tet2/3*^{DM} larvae

Bisulfite sequencing of the region upstream of the transcriptional start site for *runx1*, *scl* and *gata2b*. DNA was isolated from FACs sorted *kdrl:mCherry* positive cells at 28 hpf. Open circles indicate unmethylated CpGs, filled circles indicate CpGs in which

the cytosine residue carries either the methyl or hydroxymethyl modification. Each horizontal row indicates an individual bisulfite clone.

Notch signaling allows for communication between cells in close proximity. The ligand secreted by the donor cell bind to the receptor of the recipient cell, inducing a series of proteolytic cleavages of the Notch receptor that release the Notch intracellular domain (NICD). Once released, NICD enters the nucleus and, together with transcriptional partners such as RBPjK and co-activator Mastermind, stimulates the transcription of Notch target genes (Bray, 2016). The communication between cells through Notch signaling is required for many tissue specification, lineage commitment, and cell maintenance events throughout development. Among the many developmental processes, Notch signaling is especially important to hematopoiesis.

To test whether mutation of *tet2/3* disrupted Notch signaling in the hemogenic endothelium, we introduced the *tet2* and *tet3* mutant alleles into a Notch reporter line, *Tp1:GFP*, which expresses GFP under the control of tandem Notch responsive elements (Parsons et al., 2009). At the whole embryo level, wildtype and *tet2/3*^{DM} larvae exhibited similar patterns of GFP expression from the *Tp1:GFP* transgene, suggesting that Notch signaling was not globally compromised by mutation of *tet2/3 (*Figure 12A-12D).

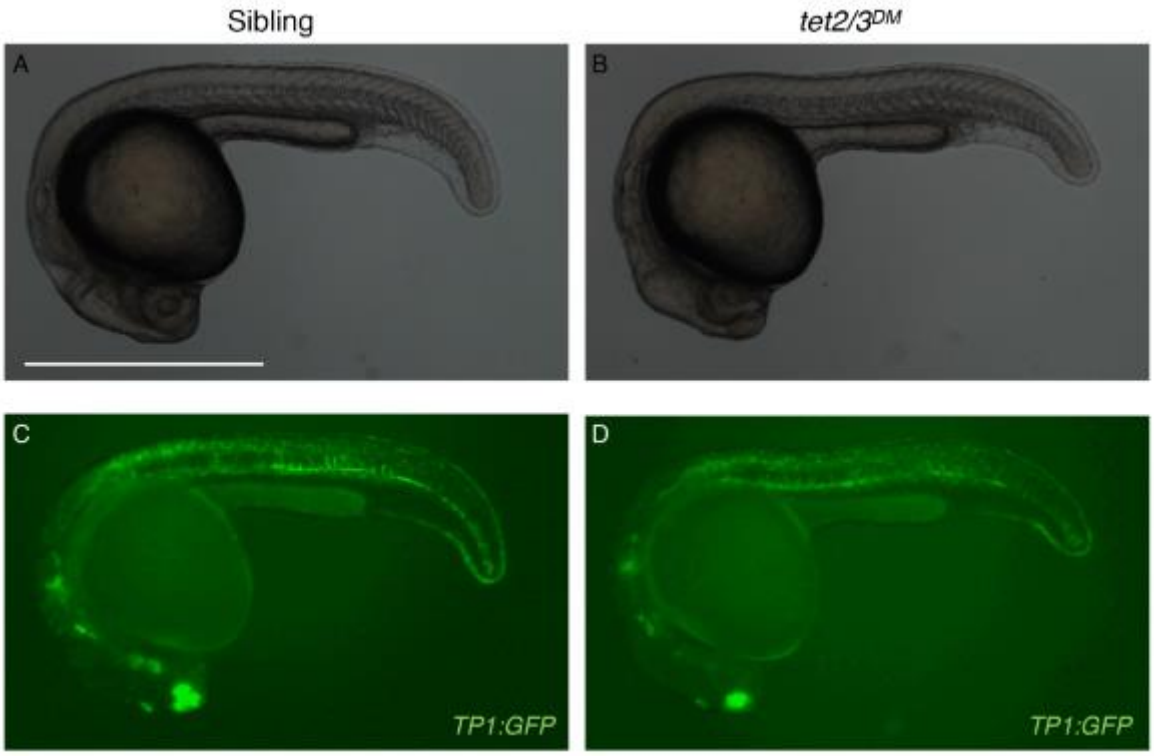


Figure 12. *Tp1:GFP* expression reveals similar overall patterns of Notch signaling in sibling and *tet2/3*^{DM} larvae

(A-B) Bright-field images of a representative sibling and *tet2/3*^{DM} larvae at 26 hpf. (C-D) GFP expression in *Tp1:GFP* transgenic larvae at 26hpf.

At higher resolution, morphologically wild-type embryos carrying the *TP1:GFP* transgene exhibited the expected strong expression of *Tp1:GFP* along both the dorsal and ventral walls of the DA (Figure 13A). In *tet2/3*^{DM} larvae, *Tp1:GFP* expression on the dorsal side of the DA appeared similar to wild type, however, expression along the ventral wall appeared weaker and discontinuous (n=10/11, Figure 13B). Quantification of mean GFP fluorescent intensity in each region revealed a 3-fold reduction in the ratio of GFP fluorescence in the ventral DA compared to the dorsal DA in *tet2/3* double mutants (p=0.0002, Figure 13C). This specific reduction in GFP in the ventral

DA reveals a requirement for Tet2/3 in regulating Notch signaling in the hemogenic endothelium and provides a mechanistic explanation for the down regulation of *gata2b* observed in *tet2/3*^{DM} larvae.

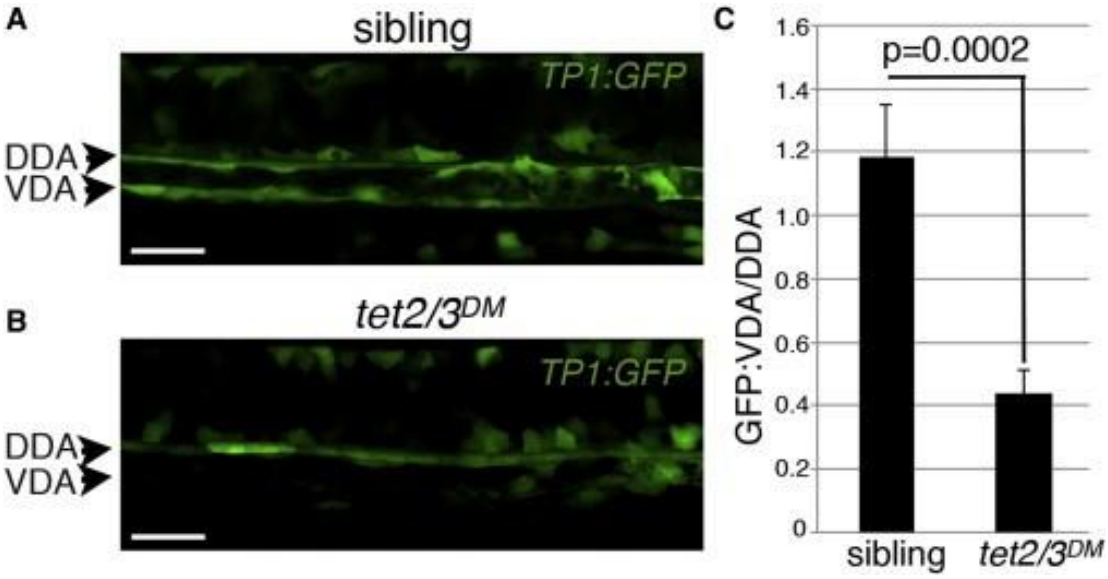


Figure 13. Tet2/3 are required for Notch signaling in the hemogenic endothelium

(A-B) Confocal images of *Tp1:GFP* expression in the dorsal aorta of sibling and *tet2/3*^{DM} larvae at 28 hpf. DDA indicates the dorsal wall of the dorsal aorta. VDA indicates the ventral wall of the dorsal aorta. (C) Ratio of GFP fluorescence intensity in the VDA/DAA in sibling and *tet2/3*^{DM} larvae (n=11 per genotype). Numerical data is presented as the mean ± SEM.

To examine whether Notch signaling is sufficient to rescue impaired HSC development in tet2/3 double mutants, we overexpressed NICD within the vascular endothelium. The *kdrl:miniGAL4* transgenic line drives the expression of transcriptional activator GAL4 in *kdrl*+ cells, while the *UAS:NICD-myc* transgenic line allows for the expression of NICD when

transcriptional activators bind to the regulatory elements UAS. Thus, *kdrl:miniGAL4* and *UAS:NICD-myc* double transgenic lines enable restricted NICD expression to the *kdrl*+ cells enriched in the vascular endothelium (Kim et al., 2014). This pattern was confirmed by immunostaining against *myc* tag, which revealed the vascular endothelial cells only in individuals with both transgenes exhibited elevated NICD expression (Figure 14A-14B).

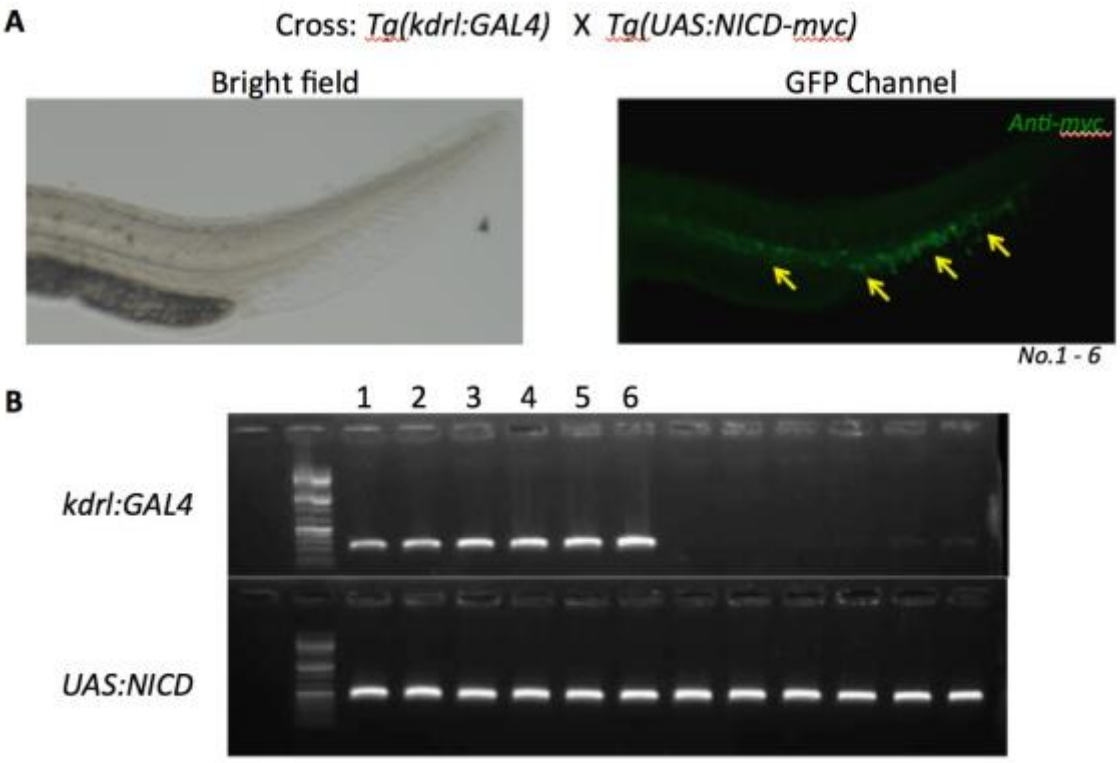


Figure 14. *Kdrl:miniGAL4* and *UAS:NICD-myc* double transgenic lines enable restricted NICD expression in the vascular endothelial cells

(A). Bright-field image and *myc* expression of a representative larva (one out of six) carrying both *kdrl:miniGAL4* and *UAS:NICD-myc* at 30 hpf. Yellow arrows indicate the restricted expression of *myc* in the vascular endothelial cells. (B) Genotype of twelve larvae tested (number 1 to 6: larvae carrying both *kdrl:miniGAL4* and *UAS:NICD-myc*).

We next introduced *tet2* and *tet3* mutations into *kdrl:miniGAL4* and *UAS:NICD-myc* transgenic lines to obtain *kdrl:miniGAL4;tet2*$^{mk17/+}$*;tet3*$^{mk18/+}$ and *UAS:NICD-myc;tet2*$^{mk17/+}$*;tet3*$^{mk18/+}$*,* respectively*. Runx1* and *c-myb* WISH were performed on the embryos derived from the intercross, and the genotype of the individual embryo was subsequently confirmed by PCR genotyping. While mutants carrying none or only one of the transgenes (5/38) lacked HSCs as before (5/38), *runx1* expression was rescued in the DA of double mutant larvae carrying both transgenes at 32 hpf (6/6) (Figure 15A-15C). Similar to the *runx1* expression, the *c-myb* expression was also observed in double mutant larvae carrying both transgenes at 32 hpf (Figure 15D-15E). However, the *c-myb* expression in *tet2/3*^{DM} larvae was lost by 3 dpf, suggesting Notch signaling is not sufficient to support the HSPCs after they are specified in the *tet2/3*^{DM} larvae (Figure 15F-15G).

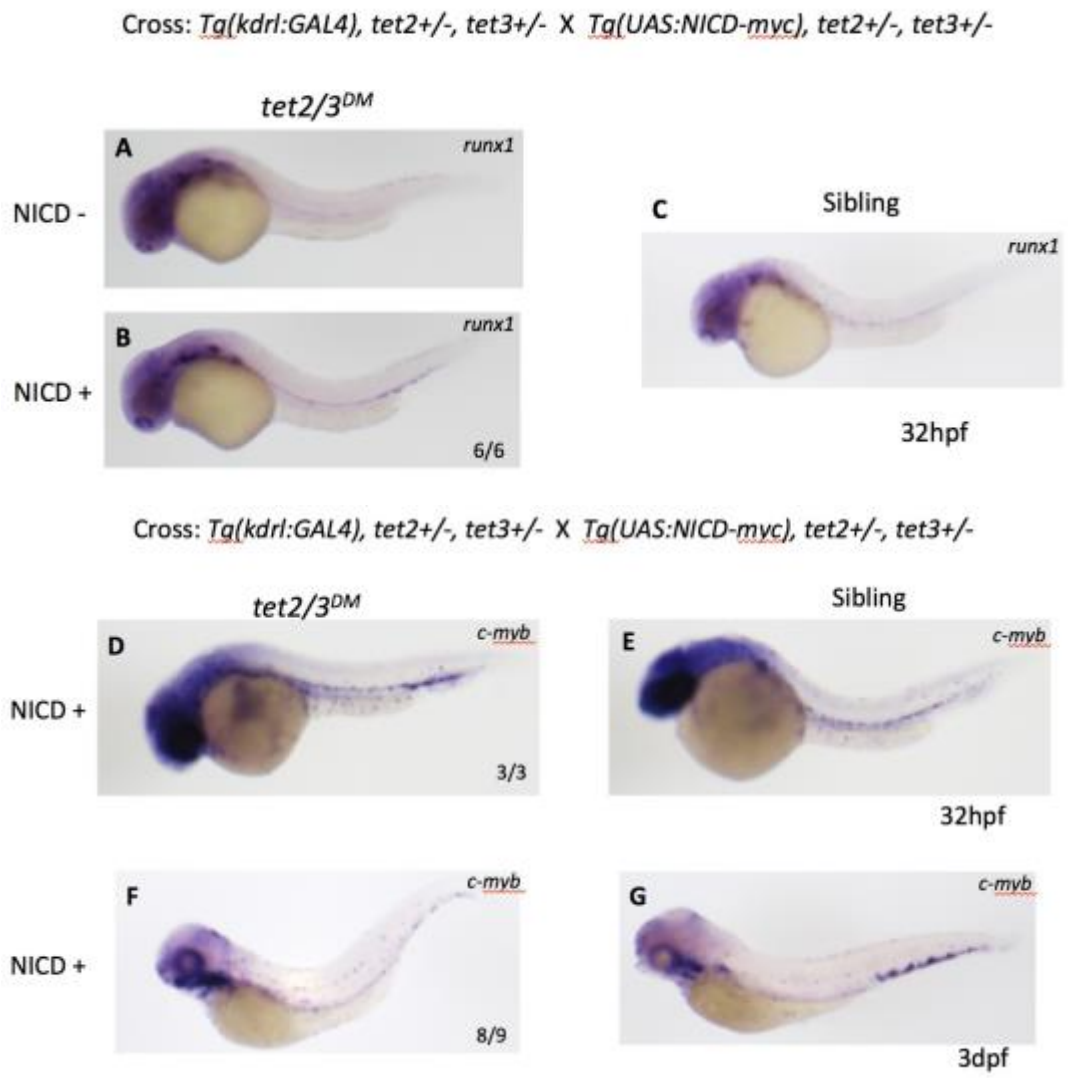


Figure 15. Overexpressing Notch signaling rescues the *runx1*+ and *c-myb*+ cells in *tet2/3*^{DM} larvae at 32hpf

(A-C) WISH for the HSC marker *runx1* at 32 hpf. (D-E) WISH for the HSC marker *c-myb* at 32 hpf. (F-G) WISH for the HSC marker *runx1* at 3dpf.

Taken together, these results uncover a requirement for Tet2/3 in the early function of the hemogenic endothelium and identify Notch signaling and the

downstream expression of the *gata2b/scl/runx1* transcriptional network as key targets of Tet2/3 regulation during HSC emergence.

Section 3: Altered inflammatory signaling is associated with impaired primitive hematopoiesis in *tet2/3^{DM} larvae*

3.1 Inflammatory signaling genes are down-regulated in the vascular endothelial cells of tet2/3^{DM} larvae

To clarify mechanisms that might act upstream of Notch signaling that contribute to the HSC phenotype in *tet2/3^{DM}* larvae, we performed massively parallel RNA sequencing on isolated vascular endothelial cells prior to the onset of EHT. The *kdrl:mCherry* transgenic line enables the isolation of mCherry+ cells enriched for vascular endothelial population by fluorescent activated cell sorting. We set the stage at 26 hpf for cell sorting as it was the earliest time we could confidently distinguish the *tet2/3^{DM}* from siblings. The hemogenic endothelium had also been specified at 26 hpf (Zhen et al., 2013). The isolated mCherry+ cells from mutants and siblings were subsequently used for RNA sequencing (40 millions reads, 100bp paired ends). Using a threshold of FDR < 0.05 with $Log_2FC > 1$ or $Log_2FC < -1$, the RNA sequencing results showed 101 genes were up-regulated and 214 genes were down-regulated in the mCherry+ cells from *tet2/3^{DM}* larvae compared

with siblings (Figure 16). GO analysis revealed that a large number of these genes (25%) were involved in inflammatory signaling (Figure 17).

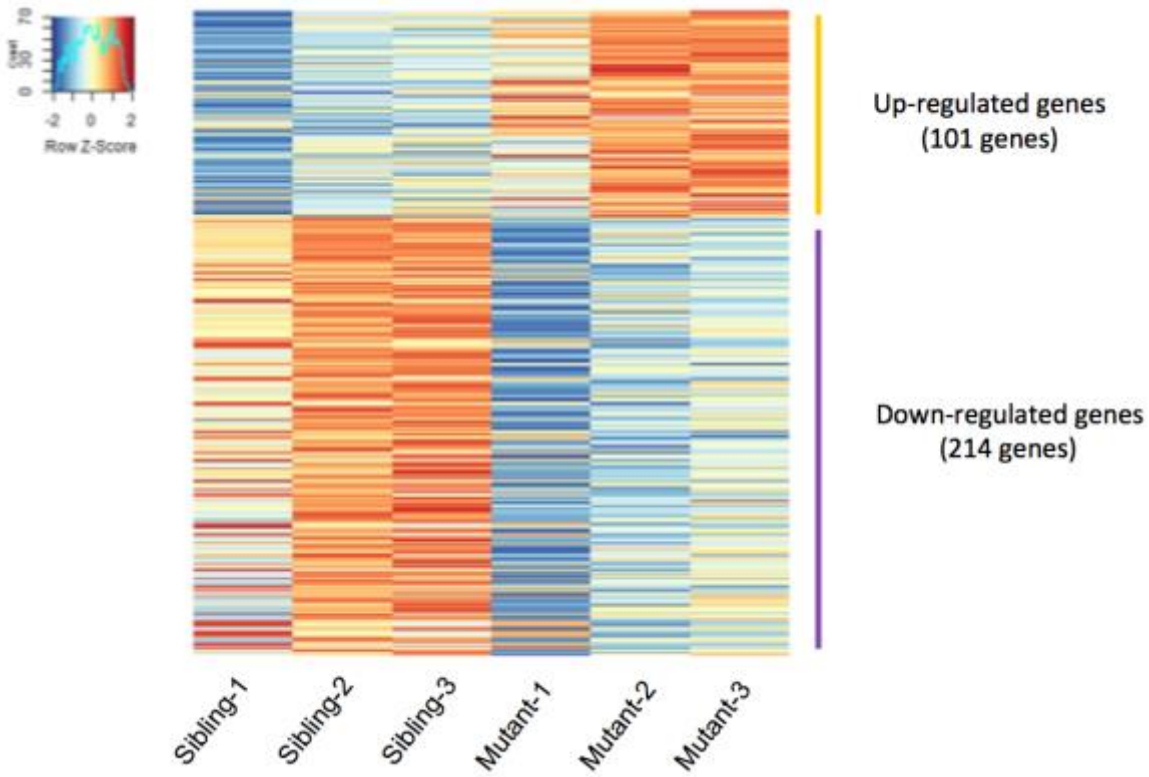


Figure 16. Heatmap of 315 genes that are differentially expressed in the mutant compared to the siblings with fold change greater than 1 and adjusted p-value less than 0.05.

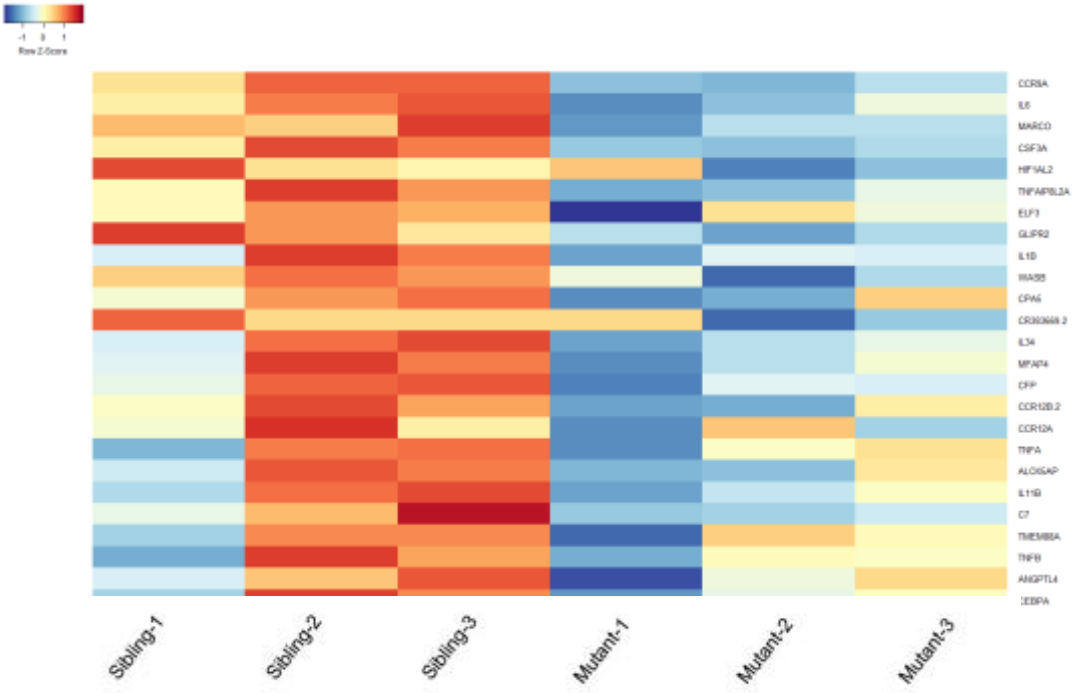


Figure 17. Heatmap of the most down-regulated inflammatory genes in the mutant compared to the siblings using supervised analysis. Figure lend on the right indicates the corresponding gene names.

Enrichment for inflammatory genes was of interest as recent studies have identified connections between inflammatory signaling and development of the hemogenic endothelium. Inflammatory signaling molecules are highly enriched in hemogenic endothelium both in zebrafish and mammals (He et al., 2015). In particular, IFNγ and its receptor act cell-autonomously to control EHT transition downstream of Notch signaling (Sawamiphak et al., 2014). Inflammatory cytokines such as TNFα have also been shown to stimulate Notch activity in the hemogenic endothelium through the Notch receptor (Espín-Palazón et al., 2014, He et al., 2015; Li et al., 2014), contributing to the establishment of the transcriptional network for EHT. Supported by these

studies, our RNA sequencing data revealed the HSC phenotypes in the *tet2/3*^{DM} larvae could be attributed to the loss of inflammatory signaling.

3.2 Abnormal development of primitive myeloid cells in tet2/3^{DM} *larvae*

Once source of inflammatory cytokines are primitive neutrophils. These signals have been shown to act in a non-cell autonomous manner to regulate the hemogenic endothelium (Espín-Palazón et al., 2014; He et al., 2015). Our initial analysis noted that early markers of primitive hematopoiesis were normal in *tet2/3*^{DM} larvae as assessed by expression of *gata1*, *scl* and *pu.1* at 25 hpf by WISH (Figure 18A-18I).

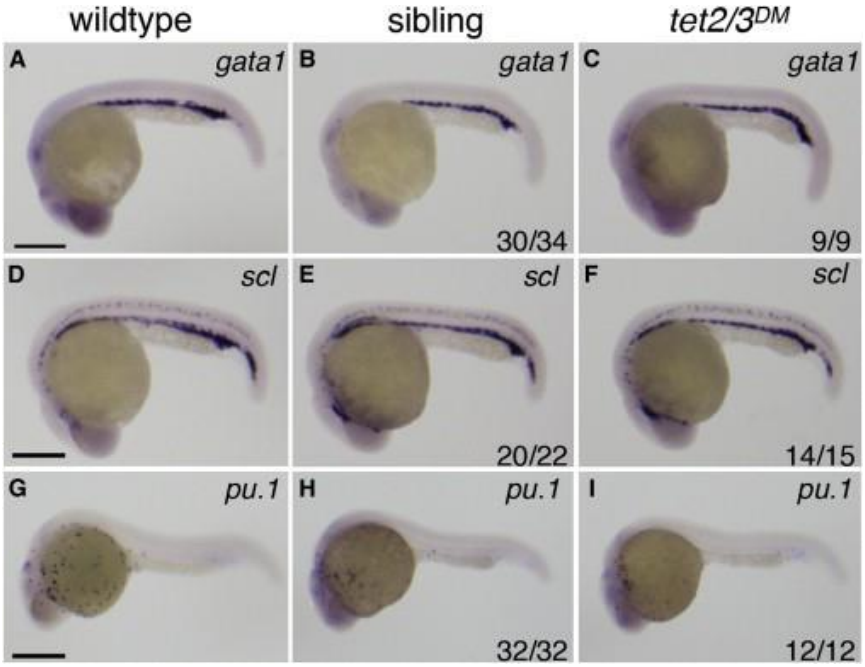


Figure 18. Primitive markers are similarly expressed in the wildtype, *tet2*$^{mk17/mk17}$ and *tet2/3*^{DM} larvae.

(A-I) Representative lateral views of wild-type larvae compared to *tet2/3*^{DM} and *tet2*$^{mk17/mk17}$ sibling larvae derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses. (A-C) WISH for the primitive erythroid marker *gata1* at 25 hpf. **(**D-F**)** WISH for the primitive erythroid marker *scl* at 25 hpf. **(**G-I**)** WISH for the primitive myeloid marker *pu.1* at 25 hpf

However, our RNA sequencing data indicated that other genes associated with myeloid differentiation were deregulated in *tet2/3*^{DM} larvae. In particular, we noted that *csf3a* and *csf3b,* encoding GCSF proteins, were significantly down regulated (Figure 17). This family of glycoproteins are known to stimulate the proliferation, differentiation, and function of myeloid precursors and mature myeloid cells, suggesting that their might be defects in myeloid cell development in *tet2/3*^{DM} larvae.

Intriguingly, csf3a/3b was previously shown to expand the HSC population in zebrafish, while morpholino knock-down caused significant decreases in HSCs (Stachura et al., 2013). To evaluate whether down-regulation of csf3a/3b contributes to the HSC phenotype in the *tet2/3*^{DM} larvae, we injected embryos from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses with mRNAs encoding Csf3a or Csf3b. We found that overexpressing either gene rescued the *runx1* expression in the *tet2/3*^{DM} (Figure 19). The data suggest that overexpression of *csf3a/3b* is sufficient to rescue the HSC phenotype observed in the *tet2/3*^{DM}*.*

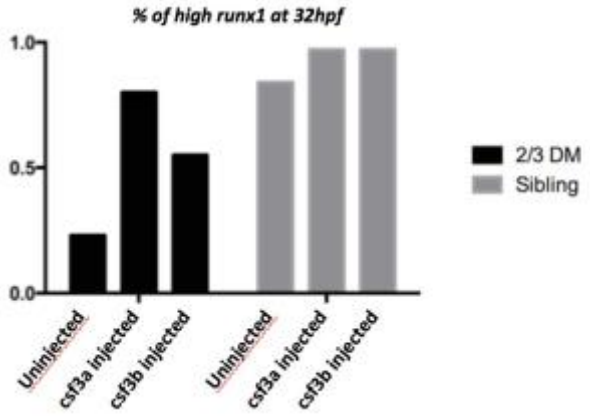


Figure 19. Quantification of WISH for *runx1* in siblings and *tet2/3*^{DM} injected with wither *csf3a* or *csf3b* RNAs

Given the involvement of csf3a/b in regulating myeloid cell maturation and the known link between myeloid mediated inflammatory signals and HSC development, we set out to examine whether primitive myeloid populations are affected in the *tet2/3*^{DM} larvae. Although pu.1, marker of undifferentiated primitive myeloid cells appeared similar in wild type and mutants, we noted that, *mfap4,* which specifically label primitive macrophages, was down regulated (Figure 20D-20F). By performing WISH for primitive neutrophil marker *mpx*, we found primitive neutrophil population remained largely unchanged in the *tet2/3*^{DM} (Figure 20A-20C).

Taken together, our RNA sequencing analysis and genetic studies are consistent with a model in which inflammatory signaling acts upstream of Notch signaling to control the HSC development. We hypothesize that reduced *csf3a/3b* expression in the *tet2/3*^{DM} negatively affects the primitive

myeloid cell population causing disruption of inflammatory signaling required for proper development of the hemogenic endothelium. To confirm the epistasis of inflammatory signaling and Notch signaling, future experiments should examine whether overexpressing csf3a/3b leads to restored Notch activity in the hemogenic endothelium of the *tet2/3*^{DM}. To study whether impaired primitive myeloid cells directly contribute to the HSC phenotypes in *tet2/3*^{DM} larvae, we could theoretically transplant wild-type primitive macrophages to the *tet2/3*^{DM} and evaluate the expression of HSC markers.

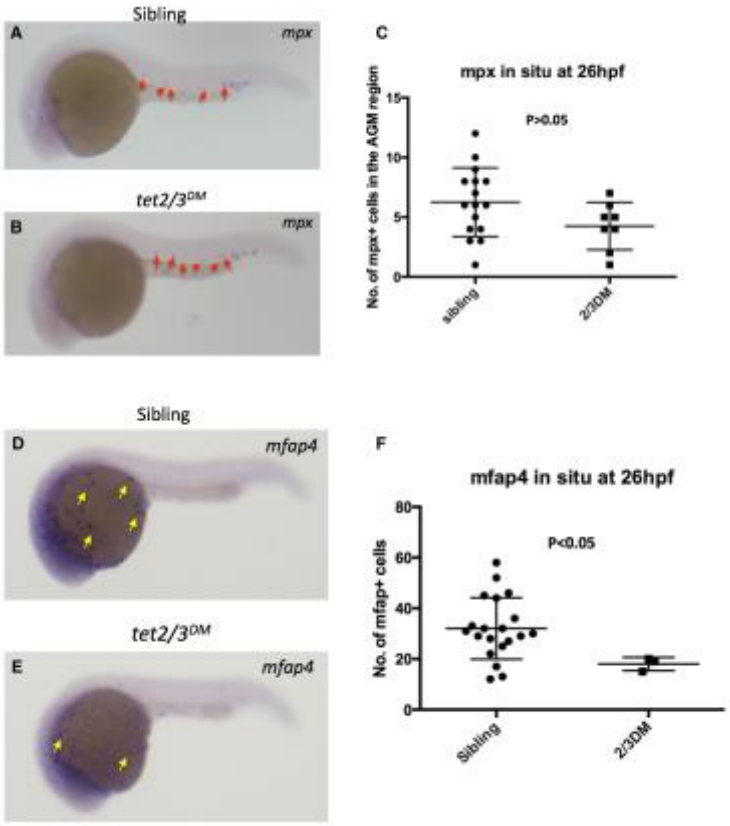


Figure 20. *tet2* and *tet3* are required for the development of primitive macrophages

(A-B) WISH for the primitive mutrophils marker mpx at 26hpf. The red arrow indicates the *mpx*+ cell in the AGM region. (C) Quantification of the number of *mpx*+ cells in the AGM region. (D-E) WISH for the primitive macrophages marker mfa4 at 26hpf. The yellow arrow indicates the representative *mfap4*+ cell on top of the yolk sac. (F) Quantification of the number of *mfap4*+ cells on top of the yolk sac.

Section 4. Enhanced Tet1 activity can compensate for loss of Tet2 and Tet3 during HSC emergence

4.1 Overexpressing mRNA encoding Tet1 rescues HSC development in tet2/3^{DM} larvae

All TET family members share highly conserved catalytic domains at the C-terminus, allowing them to execute successive oxidation of 5mC to various oxidized derivatives. However, the extent to which TET family members can compensate for each other remains unclear. Our genetic analysis has demonstrated that there are overlapping requirements for Tet2 and Tet3 during the HSC development in zebrafish, but Tet1 is dispensable for this process. However, we find that *tet1* is expressed at significantly lower levels than *tet2* and *tet3* in whole embryos and in the *kdrl* positive vascular endothelial cells at the relevant stages (Figure 21A-21B). These observations suggest that it may be the relative abundance of particular TET enzymes rather than an inherent specificity that dictates their requirements. To test this hypothesis, we asked whether overexpression of mRNA encoding Tet1 could rescue HSC phenotypes in *tet2/3^{DM}* larvae.

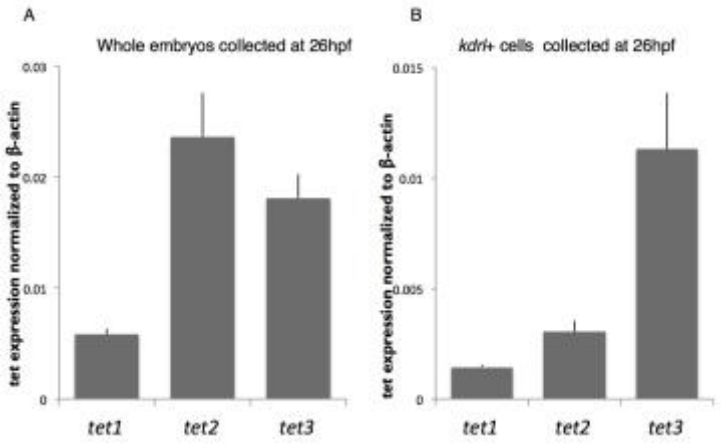


Figure 21. *tet* expression in whole embryos and *kdrl*+ cells at 26hpf

(A) qRT-PCR analysis of *tet* expression in whole embryos collected at 26hpf. (B) qRT-PCR analysis of *tet* expression in kdrl+ cells sorted at 26hpf. Numerical data is presented as the mean ± SEM from three independent groups.

To this end, we injected mouse *Tet1* RNA into the zebrafish embryos derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses. Overexpression of *Tet1* RNA in wild-type embryos did not cause developmental abnormalities with the exception of a tail blister, which occurred in 26% of injected embryos (Figure 22A-22B, 22D)(Figure 22C, 22E). However, over expression was able to rescue *runx1*+ HSCs in *tet2/3*^{DM} larvae (Figure 22G-22H). Consistent with this rescue, we noted that overexpressing *Tet1* RNA partially increased 5hmC levels in *tet2/3*^{DM} larvae (Figure 22F). These data suggest that the overexpression of Tet1 can compensate the loss of Tet2 and Tet3 during the development of HSCs. Our results provide evidence that members of Tet family behave similarly and can act interchangeably when expressed at sufficient levels.

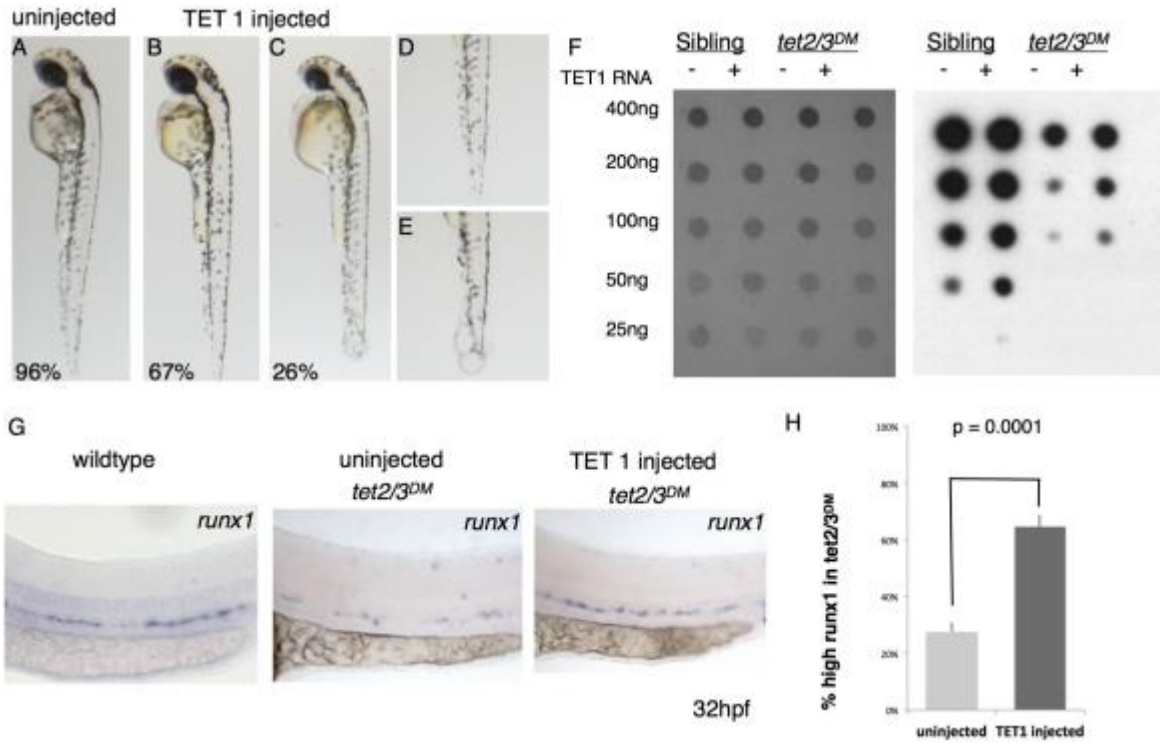


Figure 22. Overexpressing *Tet1* RNA can compensate the loss of Tet2 and Tet3

(A-E) Pictures of representative larva of control groups and *Tet1* RNA injected groups. Numbers in the lower left hand corner indicate the fraction of embryos with morphology similar to the representative image. (F) Methylene blue staining and dot blot for 5hmC on genomic DNA isolated from larvae at 32hpf. (G) WISH for the HSC marker *runx1* at 32hpf. (H) Quantification of the percent of *tet2/3*^{DM} larvae exhibiting high *runx1* staining in uninjected and *Tet1* RNA injected group. Numerical data is presented as the mean ± SEM.

4.2 Stimulation of endogenous Tet1 activity can rescue the HSC phenotypes in tet2/3^{DM} *larvae*

TET proteins can be either regulated at transcriptional level by modulating *Tet* expression or at the level of activity by various protein modifications or the presence of co-factors. One such co-factors, vitamin C, is a potent antioxidant and has been shown to enhance TET activity in ES cells with an increase in

the global 5hmC content. Compared with other reducing agents, Vitamin C can uniquely interact with the C-terminal catalytic domain of TET enzymes, promoting the recycling of iron during the oxidation processes. The capacity of vitamin C to robustly induce TET activity has also been shown across a wide spectrum of cells, including mouse embryonic fibroblasts, T regulatory cells, and melanoma cells. However, this capacity has not yet been tested in an in vivo model. Our zebrafish model provides a promising platform to test the in vivo potency of vitamin C to stimulate TET enzymatic activity.

We set out to examine whether vitamin C treatment could stimulate Tet1 activity and whether it was sufficient to rescue HSC development in *tet2/3^{DM}larvae.* We found that vitamin C treatment did not impact overall development of zebrafish larvae at tested concentrations (1mM), nor did it affect the endogenous *tet* expression (Figure 23A-E). However, supplementing vitamin C resulted in a dose-dependent increase in the 5hmC levels in both siblings and *tet2/3^{DM} larvae* (Figure 24F). In addition, we found that *runx1*+ cells were rescued in the *tet2/3^{DM}* when treated with vitamin C (Figure 24A-24E). To confirm that the rescue of *runx1* expression was mediated through the stimulation of Tet1, we tested whether rescue was still possible in *tet1/2/3^{TM}* larvae. Using the same dose of vitamin C, *runx1*+ cells were not detected in triple mutants, suggesting that rescue was mediated through Tet1 stimulation (Figure 24G-24H).

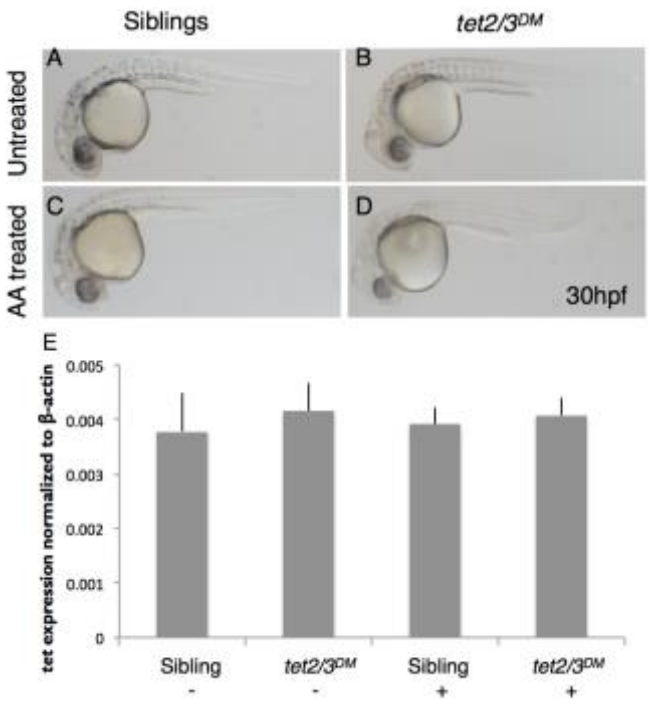


Figure 23. Vitamin C (AA) treatment minimally affects the morphology and the expression of tet1 in siblings and *tet2/3*^{DM}

(A-D) Representative pictures of siblings and *tet2/3*^{DM} either untreated or treated with 1mM Vitamin C. (E) qRT-PCR analysis of *tet1* expression in siblings and *tet2/3*^{DM} either untreated or treated with 1mM Vitamin C. Numerical data is presented as the mean ± SEM from three independent experiments.

Taken together, these results confirm the in vivo potency of vitamin C to stimulate TET enzymatic activity. More importantly, our study shows the HSC defects in the *tet2/3*^{DM} can be rescued by stimulating endogenous Tet1 activity via vitamin C treatment, suggesting there is the potential to use vitamin C to treat TET-related diseases. Our system also sets the stage for screens for additional factors that rescue the HSC defects, which may potentially regulate Tet1 activity or expression.

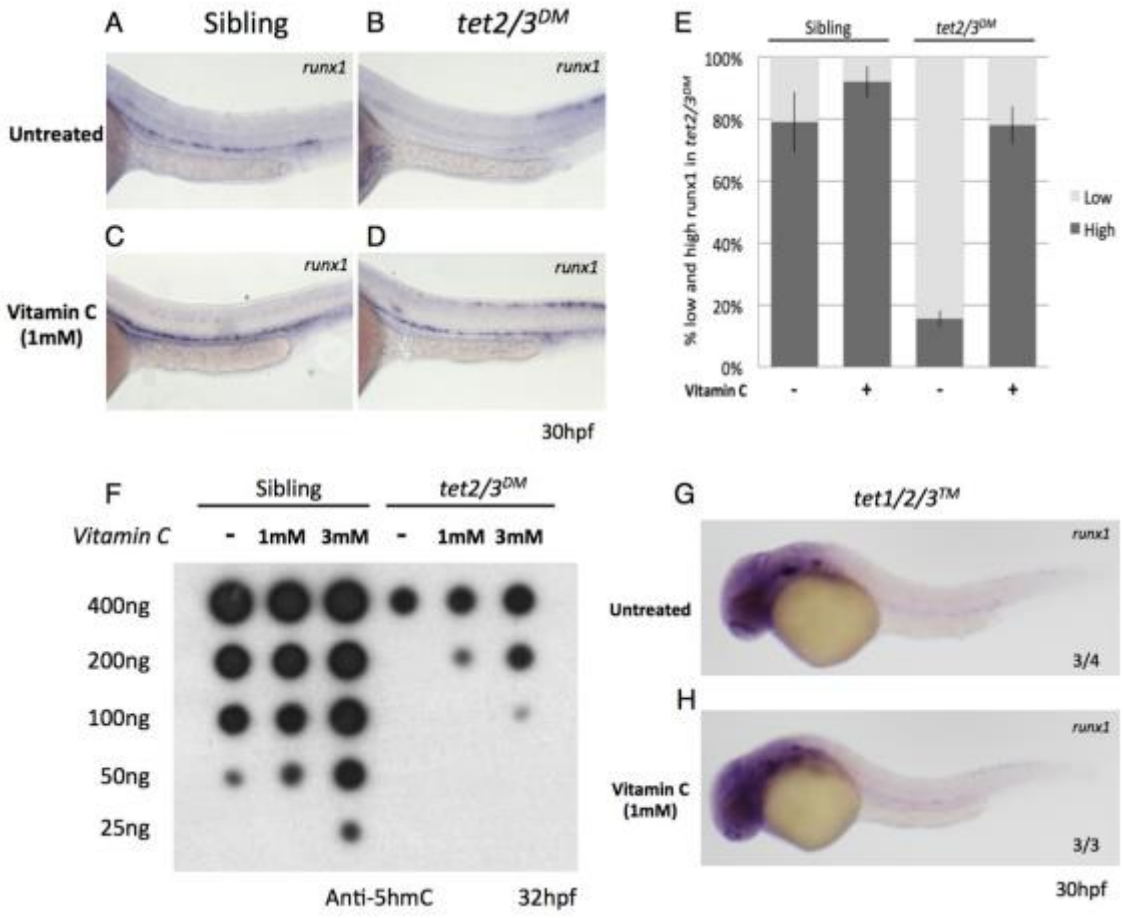


Figure 24. Vitamin C treatment rescues the HSC phenotype in *tet2/3*^{DM} larvae

(A-D). WISH for the HSC marker *runx1* at 30hpf in siblings and *tet2/3*^{DM} either untreated or treated with 1mM Vitamin C. (E) Quantification of the percent of *tet2/3*^{DM} larvae exhibiting high *runx1* staining in siblings and *tet2/3*^{DM} either untreated or treated with 1mM Vitamin C. Numerical data is presented as the mean ± SEM. Pictures in panel A-E were prepared by Lianna Schwartz-Orbach. (F) Dot blot for 5hmC on genomic DNA isolated from larvae at 32hpf treated with 1mM or 3mM Vitamin C. (G, H) WISH for the HSC marker *runx1* at 30hpf in siblings and *tet1/2/3*^{TM} either untreated or treated with 1mM Vitamin C. Numbers in the lower right hand corner indicate the fraction of embryos exhibiting WISH labeling similar to the representative image.

Section 5. Applications of using *the tet2/3^{DM}* as a platform to study novel regulators of TET proteins

5.1 Identifying suppressors of HSC loss in tet2/3^{DM} larvae

In section 4, we demonstrated that either overexpressing Tet1 mRNA or stimulating endogenous Tet1 activity rescues HSC defects in *tet2/3^{DM}* larvae. Based on these observations, we reasoned that *tet2/3^{DM}* larvae could be used as a platform to identify additional modulators of Tet1 activity. As a first approach, we performed a pilot small molecule suppressor screen designed to identify small molecules that stimulated Tet1 expression or activity. To this end we used the Selleck Epigenetics Compound library, which contains 181 small molecule modulators with biological activity in epigenetic regulation. Groups of an average 20 embryos derived from intercrosses between *tet2$^{mk17/mk17}$, tet3$^{mk18/+}$* adults were treated with compounds from this library at a concentration of 15mM beginning at 8hpf. Treated embryos and untreated controls were then collected at 30hpf and assayed for *runx1* by WISH. Compounds that resulted in higher expression level of *runx1* in double mutants compared with untreated double mutant controls were identified as candidates for additional analysis.

Using this approach, we identified three drugs SGI-1776, M344, and LAQ824 that were able to reproducibly rescue the *runx1* expression in the *tet2/3^{DM}*

larvae. These compounds included the PIM kinase inhibitor SGI-1776 and the histone deacetylase (HDAC) inhibitors M344 and LAQ824.

PIM kinases are a small family of serine/threonine kinases regulating several signaling pathways that are fundamental to cancer development and progression (Swords et al., 2011). HDACs are a class of enzymes that remove acetyl groups from a histone, an activity associated with transcriptional repression (Haberland, Montgomery, & Olson, 2009). Based on their mechanism of action, we tested the likely hypothesis that HDAC inhibition caused elevated levels of *tet1* transcription in *tet2/3*^{DM} larvae. However, we did not detect statistically significant changes in *tet1* expression in treated larvae (Figure 25), suggesting that either *tet1* upregualtion was only in a subset of cells diluted out in our whole embryo analysis or that other mechanism mediate the suppression of HSC loss in *tet2/3*^{DM} larvae. For example, HADC inhibition may directly derepress genes involved in the HSC developmental program.

Although additional follow up will be required, the results of this preliminary chemical suppressor screen, suggest that this approach may be a promising application of the *tet2/3*^{DM} larvae.

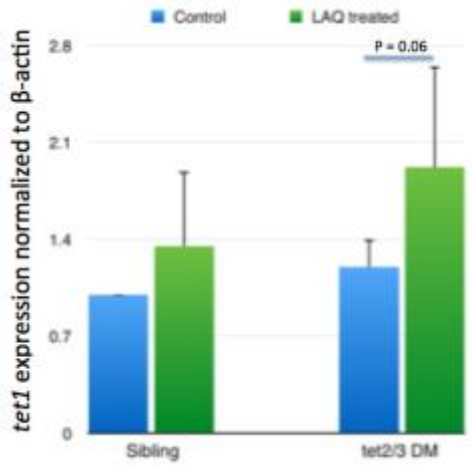


Figure 25. LAQ824 treatment does no increase *tet1* expression. Numerical data is presented as the mean ± SEM from five independent experiments.

5.2 Clarifying the importance of TET-OGT interactions using tet2/3^{DM} larvae

The *tet2/3*^{DM} larvae also offer an exciting platform for in vivo structure/function analysis of TET mutant proteins. Validating this approach, in collaboration with the Panning lab (UCSC) we have investigated the importance of interactions between Tet1 and *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OGT). Previous studies have demonstrated that OGT catalyzes the addition of *O*-GlcNAc to serine and threonine residues in a wide range of proteins (Bond & Hanover, 2015). Its activity depends on the availability of various metabolic molecules such as glucose, ATP, glutamine, and acetyl-CoA (Harwood & Hanover, 2014). OGT has been shown to stably interact with all three Tet proteins, and the interaction of OGT with TET proteins has been reported to influence histone modifications and gene expression (Bauer

et al., 2015; Chen et al., 2012; Deplus et al., 2013; Shi et al., 2013; Vella et al., 2013). However, the mechanism of the TET-OGT interaction is unclear and how this interaction affects TET enzymatic activity in vivo remains an open question.

Given that Tet1 can compensate the loss of Tet2 and Tet3 during the emergence of HSCs, we asked whether a Tet1 mutant protein with impaired TET-OGT could to rescue the HSC phenotypes in *tet2/3*^{DM} larvae. The Panning lab mapped the interaction between TET1 and OGT to a small C-terminal region of TET1 and demonstrated that OGT binding enhances the catalytic activity of TET1 in vitro. As part of a collaboration, we asked whether the potential for Tet1 to rescue HSC phenotypes in *tet2/3*^{DM} larvae was also dependent on its capacity to interact with OGT. Mutation in D2018 of TET1 was known to abolish its OGT interaction capacity in vitro. To test whether this mutation also abolished in vivo activity, embryos derived from intercrosses between *tet2*$^{mk17/mk17}$*, tet3*$^{mk18/+}$ adults were injected with either D2018A mutant *Tet1* RNA or wild-type *Tet1* RNA. The embryos were subsequently collected at 30hpf and assessed for *runx1 by* WISH. We found that while overexpressing wild-type*Tet1* RNA significantly increased *runx1* expression in the DA of *tet2/3*^{DM} larvae, overexpressing *Tet1* D2018A mRNA failed to rescue *runx1 expression* in double mutants (Figure 26A-26B).

Consistent with this observation, we noted that the *tet2/3*^{DM} *larvae* injected with wild-type *Tet1* RNA exhibited a modest increase in the global 5hmC levels relative to the uninjected controls, however, the elevated global 5hmC levels were lost in the *tet2/3*^{DM} injected with D2018A mutant *Tet1* RNA (Figure 26C). Overall, these data suggest that TET-OGT interaction is required for Tet1 to compensate the loss of Tet2 and Tet3 in the HSC development.

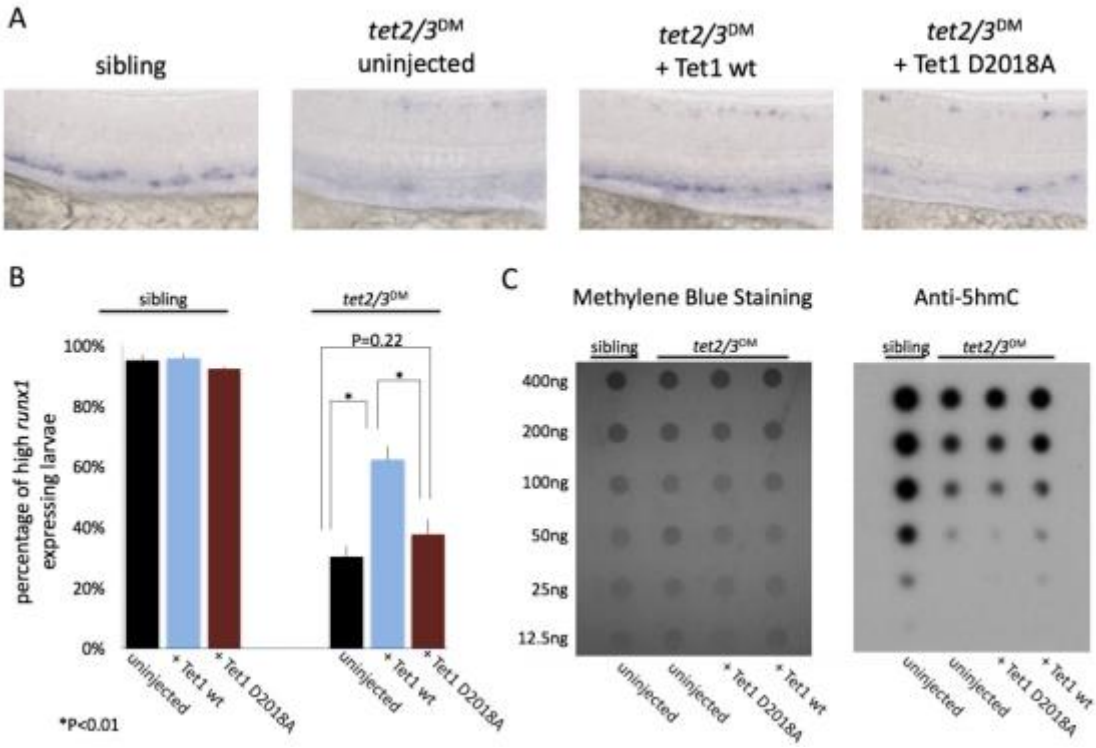


Figure 26. The interaction with OGT is required for TET1 activity in the zebrafish embryo

(A) WISH for the HSC marker *runx1* expression in the dorsal aorta of wild type or *tet2/3*^{DM} zebrafish embryos, uninjected or injected with mRNA encoding Tet1 wild type or D2018A. (B) Percentage of embryos with high *runx1* expression alongthe dorsal aorta. (C) Methylene blue staining and dot blot for 5hmC on genomic DNA isolated from larvae at 32hpf.

CHAPTER THREE: DISCUSSION

1.1 TET requirements in normal zebrafish development

Our systematic analysis of *Tet* mutant phenotypes revealed Tet2 and Tet3 to be the major 5mC dioxygenases in the zebrafish embryo. To date, a number of studies in mouse have focused on *Tet1* and *Tet2*, likely due to the fact that these are the only *TET* orthologs expressed in ESCs (Wu and Zhang, 2011). Nonetheless, *Tet3* is up regulated upon differentiation of ESCs and is highly expressed in many differentiated primary tissues (Dawlaty et al., 2013; Li et al., 2015). A more recent study showed that acute deletion of Tet2 and Tet3 in hematopoietic compartment leads to a more aggressive myeloid leukemia phenotype compared to *Tet2* single mutant mice (An et al., 2015). Our analysis of zebrafish *tet* mutants, combined with the phenotype of conditional *Tet2/3* mutant mice and the poor differentiation capacity of *Tet1/2/3* mutant ESCs, suggest that *Tet2* and *Tet3* have important overlapping requirements in ensuring normal vertebrate development (Dai et al., 2016; Dawlaty et al., 2014).

Intriguingly, while at least *Tet3* is maternally deposited in mouse, few, if any, *tet* transcripts are detected in RNA-seq data from 2-cell stage zebrafish embryos and 5hmC is not detected in the zebrafish embryo by immunofluorescence until the bud stage (Almeida et al., 2012; Gu et al., 2011). These observations imply that mRNAs encoding the TET enzymes are

not maternally deposited in zebrafish and suggest that, in contrast to mammals, the zebrafish genome does not contain significant amounts of 5hmC prior to segmentation. Zebrafish do not undergo the same TET dependent erasure and reestablishment of global 5mC patterns observed during mammalian preimplantation development, providing one potential explanation for this distinction (Jiang et al., 2013; Potok et al., 2013). The lack of maternal deposition and limited dependency on TET enzymes during the first 24 hours post fertilization make zebrafish a powerful system for examining TET requirements in later developmental processes, including those associated with tissue specific development and differentiation.

1.2 Roles for Tet2 and Tet3 in hematopoietic stem cell emergence

A focus of this thesis was to examine requirements for Tet2/3 during embryonic stages of hematopoietic development. The earliest stages of definitive hematopoiesis also appear unaffected in *Tet2* single mutant mice and zebrafish, although diminished expression of *c-myb* but not *runx1* was observed in the dorsal aorta of *tet2* morpholino injected zebrafish embryos (Ge et al., 2014; Gjini et al., 2014; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). In genetic models, loss of *Tet2* eventually leads to an expansion of hematopoietic progenitor cells in the bone marrow and skewed myeloid differentiation (Gjini et al., 2014; Ko et al., 2011; Li et al.,

2011b; Moran-Crusio et al., 2011). However, the long latency that precedes these phenotypes suggests secondary somatic mutations may be a contributing factor. Meanwhile, acute deletion of Tet2 and Tet3 in hematopoietic precursor cells leads to a highly aggressive myeloid leukemia phenotype with 100% penetrance (An et al., 2015).

In contrast to these later hematopoietic phenotypes, we find that combined mutation of *tet2/3* causes an early loss of definitive blood cells, resulting from compromised HSC production. Importantly, abnormalities in HSC development occurred in embryos that were morphologically quite normal, and had normal expression of vascular markers. The relatively normal development of *tet2/3*^{DM} larvae supports a specific role for *tet2* and *tet3* in regulating transcription of the embryonic HSC developmental program rather than a more generalized role in regulating global transcription. The different HSC phenotypes we observed compared to the *Tet2/3* mutant mice might likely reflect different requirements for Tet2/3 at different stages during HSC development. While studies by An and colleagues conditionally mutated *Tet2* and *Tet3* in hematopoietic compartment, HSCs had been formed before *Tet2* and *Tet3* were depleted. Therefore, their system is better suited to study the requirements for Tet2/3 in regulating the proliferation and differentiation of HSCs. Our study, on the other hand, used whole animal *tet2/3* double mutant, which enabled the study of the earliest requirements for Tet2 and Tet3 in HSC development.

Compared to *tet1*, both *tet2* and *tet3* transcripts are relatively enriched in the DA of the developing zebrafish embryo (Ge et al., 2014). This expression pattern provides a potential explanation for the specific overlapping Tet2/3 requirements in HSC production. Analysis of double mutant larvae revealed a combined requirement for *tet2/3* in regulating Notch signaling in the hemogenic endothelium, suggesting a role for 5hmC in the specification or early function of this tissue. Enrichment of 5hmC has been reported at Notch receptor and ligand genes in other tissues, but the functional significance of these changes has not been determined (Terragni et al., 2014). Intriguingly, we find Notch signaling to be relatively intact in other tissues of *tet2/3*^{DM} larvae, indicating that Tet2/3 are likely be involved in fine-tuning the activation of this pathway in select cell types. Notch signaling is essential for HSC development in vertebrates, and has been implicated in both specification of the dorsal aorta and downstream HSC production (Jagannathan-Bogdan and Zon, 2013; Robert-Moreno et al., 2005). Tet2/3 appear to be dispensable for Notch regulation of arterial specification, as we observe normal expression of the arterial marker *ephrinb2* in *tet2/3*^{DM} larvae. Instead, disruption in Notch signaling in the hemogenic endothelium favors a select requirement for Tet2/3 in the regulation of HSC specification. This model is consistent with the downstream disruption of the *gata2b/scl/runx1* transcriptional network observed in *tet2/3*^{DM} larvae and our observation that reintroducing *scl* or *gata2b* mRNA can rescue HSC production in double mutants. Notably, Notch

regulation is dispensable for *scl* expression during the primitive wave of hematopoiesis, which is consistent with the normal *scl* expression observed in *tet2/3*^{DM} primitive erythrocytes (Burns et al., 2005; Kim et al., 2013). We also find that reintroducing Notch signaling in vascular endothelial cells is sufficient to rescue the *de novo* emergence of HSC in the *tet2/3*^{DM} larvae. However, we observe that Notch signaling in the kdrl+ vasculature alone is not sufficient to support the HSC pools at the later stage. It is plausible that other pathways regulated by Tet2/3 are required for the seeding, migration, or proliferation of HSCs after they are emerged from the dorsal aorta. Alternatively, the vascular niche in the caudal hematopoietic tissue may be disrupted due to the deficiency of Tet2 and Tet3, leading to the diminished HSPC population at the later stage.

1.3 Potential links between impaired primitive hematopoiesis and loss of hematopoietic stem cells in tet2/3^{DM} *larvae*

The RNA sequencing results reveal an unexpected down-regulation of inflammatory genes in the vascular endothelial cells of the *tet2/3*^{DM} prior to the onset of EHT. It has been shown that hemogenic endothelium is enriched for inflammatory signaling, and Notch signaling functions downstream of inflammatory signaling to regulate the emergence of HSCs (Espín-Palazón et al., 2014; He et al., 2015). It is likely that Tet2/3 regulate inflammatory signaling in the hemogenic endothelium to ensure the EHT process. We

demonstrate one possible way that Tet2/3 control the inflammatory signaling by facilitating the development of primitive myeloid cells, as they can act as a source of inflammatory signals.

Defects in primitive hematopoiesis following antisense morpholino depletion of *tet2* in zebrafish and impaired differentiation of primitive embryonic/yolk sac progenitors following shRNA depletion of *TET2* in human ESCs have been previously reported (Ge et al., 2014; Langlois et al., 2014). However, these results are difficult to reconcile with the normal primitive hematopoiesis observed in published mouse and zebrafish models of *Tet2* mutation (Gjini et al., 2014; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran- Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). While it is difficult to definitively address the discrepancies between these studies, both shRNA and antisense morpholino technologies can be susceptible to off target effects (Kok et al., 2015; Scherer and Rossi, 2003). Similar to other published studies of *Tet2* mutants, our initial analysis indicates an overtly normal primitive hematopoiesis in *tet2/3*^{DM} larvae as assessed by expression of *gata1*, *scl* and *pu.1* at 25 hpf by WISH. However, subsequent study reveals a subtle primitive defect in *tet2/3*^{DM} larvae as the number *mfap4*-expressing primitive macrophages is significantly less compared to siblings. This discrepancy may reflect the different populations of primitive myeloid cells marked by *pu.1* and *mfap4* probes. Combined with RNA sequencing data, we hypothesize that reduced *csf3a/3b* expression in

the *tet2/3*^{DM} negatively affects the primitive myeloid cell population. It will be interesting to study whether the functions of primitive myeloid cells are also affected in the *tet2/3*^{DM} larvae and whether Tet2/3 directly regulate the development of primitive myeloid cells

1.4 Compensation between TET proteins

Using the *tet2/3*^{DM} as a platform, we find Tet1 can compensate the loss of Tet2/3 in the development of HSC, suggesting TET family members function similarly and can compensate the loss of others when expressed at sufficient levels. Alternatively, stimulating endogenous TET enzymatic activity by supplementing cofactors can achieve similar outcomes as Vitamin C treatment rescued the HSC phenotypes in the *tet2/3*^{DM}. Indeed, previous studies have highlighted the use of Vitamin C to stimulate TET activity in a wide range of cells including mouse ES cells, embryonic fibroblasts and T regulatory cells (Blaschke et al., 2013; Dickson et al., 2013; Nair et al., 2016; Yue et al., 2016). Our animal study validates the in vivo potency of using Vitamin C to stimulate the activity of TET enzymes, which could have therapeutic implications.

For example, the loss of 5hmC has been identified as a novel epigenetic hallmark of cancer with diagnostic and prognostic implications. In melanoma, loss of 5hmC is correlated with cancer progression, while rebuilding global

5hmC levels by overexpressing *TET* suppresses melanoma growth (Lian et al., 2012). In blood cancers, *TET2* is one of the most frequent mutations, and loss of 5hmC can be observed in the blood cells of patients with *TET2* mutations. One promising approach in treating blood cancers is to restore TET activity, however, the feasibility to overexpress *TET* directly in patients remains unclear. Vitamin C treatment appears to be a tempting way to rescue the global 5hmC levels by stimulating endogenous TET activity in cancer cells. In melanoma cells, treatment with vitamin C promoted 5hmC content and decreased malignancy of metastasis (Gustafson et al., 2015). In AML cell lines, vitamin C treatment at high concentrations induced a dose- and time- dependent inhibition of proliferation (Park, 2013). At lower concentrations, Vitamin C treatment had a synergistic inhibition of AML cell proliferation with DNMTi treatment, which is likely the results of both passive DNA demethylation by DNMTi and active conversion of 5mC to 5mC by TET enzymes (Liu et al., 2016). Interestingly, while many vertebrate species are able to synthesize vitamin C, some species, such as teleost fish, primates, guinea pigs, as well as some bat and bird species have lost the capacity to synthesize it (Drouin, Godin, & Page, 2011). This makes zebrafish an ideal in vivo model to advance the application of using vitamin C as a cancer therapy. One caveat of our study is that the concentration of vitamin C we used in our experiments was the concentration in fish water, which might not be the concentration at physiological levels. Further study should measure the

physiological levels of vitamin C to better translate the study in fish into therapeutics.

1.5 Applications of using the tet2/3^{DM} as a platform to study novel regulators of TET proteins

Finally, we demonstrate that *tet2/3^{DM}* larvae are an efficient platform to study novel regulators of TET proteins. From a forward approach, we performed a chemical suppressor screen using the expression of *runx1* as the readout. The chemical screen revealed SGI-1776, M344, and LAQ824 were able to rescue the *runx1* expression in the *tet2/3^{DM}*, suggesting these chemicals might regulate the expression or enzymatic activity of endogenous Tet1. Further experiment showed that LAQ824 treatment led to a small but consistent increase in the *tet1* expression, however, the up-regulation of *tet1* was not statistically significant. The data suggest that either *tet1* upregulation was only in a subset of cells diluted out in our whole embryo analysis or that other mechanism mediate the suppression of HSC loss in *tet2/3^{DM}* larvae. The pilot chemical screen was performed at a low concentration (15mM) to ensure the overtly normal development of fish. However, there may be merit in repeating this screen at additional concentrations to identify candidate chemicals that regulate *tet1* expression or activity with different potency.

In a second approach, we tested the importance of TET-OGT interaction in regulating Tet1 enzymatic activity using our *tet2/3*^{DM} platform. Our result that wild type Tet1, but not Tet1 carrying a mutation that can impair interaction with OGT, can rescue HSC development *tet2/3*^{DM} larvae. This finding suggests that OGT regulation of TET enzymes plays an important role in development. While previous studies have shown that TET proteins can recruit OGT to specific regions of the genome, regulating the site-specific gene expression by modifying local histone coding (Chen et al., 2012; Deplus et al., 2013; Vella et al., 2013), our results revealed an additional function of the TET-OGT interaction, which is to regulate the enzymatic activity of Tet1 proteins. It will be interesting to examine whether the TET-OGT interaction is required for Tet2 and Tet3 enzymatic activity. Together, we show that the *tet2/3*^{DM} larvae we generated can be broadly used for identifying novel Tet1 regulators either from a forward or a reverse approach.

1.6 Conclusions and Significance

Collectively, the research described in this thesis uncovers a requirement for TET regulation of 5hmC in zebrafish development and the emergence of HSC. A deeper understanding of how HSC generation is regulated in vivo is expected to facilitate the in vitro production of HSCs for therapeutic purposes. Importantly, our results identify regulation of 5hmC as an additional variable to be considered in the optimization of protocols for HSC differentiation from

pluripotent progenitors. This observation may be of particular relevance given recent data highlighting the impact of cell culture conditions on global 5hmC levels (Blaschke et al., 2013; Nestor et al., 2015; Yin et al., 2013). Additionally, searching for a cure for blood cancers calls for a better system to evaluate the pathways involved in oncogenesis. Our tet2/3^{DM} system has proven to be an efficient platform to study and test regulators of TET proteins.

CHAPTER FOUR: METHODS

Zebrafish maintenance

Zebrafish maintenance and breeding were conducted under full animal use and care guidelines with approval by the institutional animal care and use committee of the Memorial Sloan Kettering Cancer Center. Embryos and adult zebrafish were raised under standard conditions at 28.5°C.

TALEN and CRISPR/Cas mutagenesis

TALEN sequences were selected using Targeter 2.0 software (Doyle et al., 2012). TAL repeat assembly was achieved using the Golden Gate assembly method and assembled repeats were integrated into the GoldyTALEN scaffold (Bedell et al., 2012; Cermak et al., 2011). Assembled vectors served as templates for *in vitro* mRNA transcription using the T3 mMessage mMachine kit (Ambion) according to manufacturer's instructions. 50-100 pg of mRNA was injected into wild-type embryos at the one-cell stage.

CRISPR/Cas system was adopted from Chen and Wente lab (Jao, Wente, & Chen, 2013). CRISPR gRNAs were designed using MacVector and incorporated in to the pT7-gRNA core. Assembled vectors served as templates for *in vitro* transcription using the T3 RNA polymerase. Cas9 RNAs were synthesized from pT3TS-nCas9n plasmid using the T3 mMessage

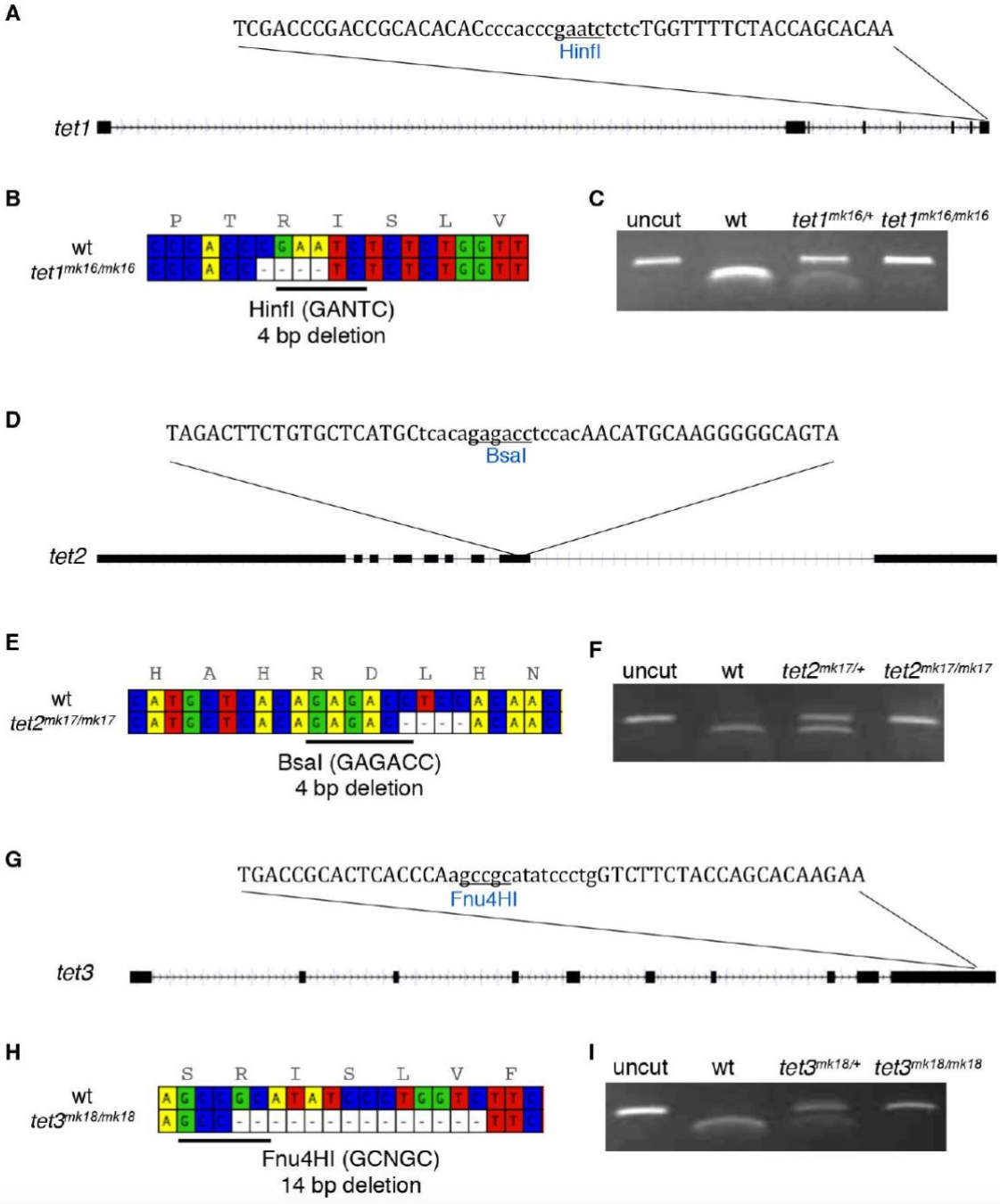
mMachine kit (Ambion). Cas9 RNAs and gRNAs were co-injected into zebrafish embryos at the one-cell stage.

Recovery of tet mutant zebrafish

Embryos injected with mRNA encoding TALEN pairs that targeted each tet gene were separately raised to adulthood and crossed to wild type. Founders carrying germline transmissible mutations were identified by isolating DNA from pooled larval progeny. DNA was PCR amplified and digested using diagnostic restriction enzymes within the region targeted for mutation. The presence of a resistant band indicated that some larval progeny from the tested founder were heterozygous for a mutation in the targeted region. Additional progeny from the identified founder were then raised to adulthood and heterozygotes were identified using the same genotyping strategy on DNA isolated from caudal fin tissue. The PCR fragment was then cloned and sequenced to identify the exact lesion. Adults carrying mutations that generated predicted frame-shift events were crossed to wild-type adults to generate stable lines (Figure 27A-27H).

Figure 27. Recovery of *tet* mutant zebrafish

(A) *tet1* TALEN design. Sequences targeted by the left and right TALEN monomers are included in capitals. The HinfI diagnostic restriction site located within the spacer region of the wild-type sequence is underlined. (B) Schematic indicating the 4 base pair deletion in the *tet1*^{mk16} allele. Amino acids encoded by the wild-type sequence are indicated at the top. The Hinf1 restriction site, which is destroyed in *tet1*^{mk16}, is underlined. (C) Representative gel genotyping larvae for the *tet1*^{mk16} mutation. Following PCR amplification of the targeted region, only the wild-type *tet1* allele can be digested by Hinf1. (D) *tet2* TALEN design. Sequences targeted by the left and right TALEN monomers are included in capitals. The BsaI diagnostic restriction site located within the spacer region of the wild-type sequence is underlined. (E) Schematic indicating the 4 base pair deletion in the *tet2*^{mk17} allele. Amino acids encoded by the wild-type sequence are indicated at the top. The BsaI restriction site, which is destroyed in *tet2*^{mk17}, is underlined. (F) Representative gel genotyping larvae for the *tet2*^{mk17} mutation. Following PCR amplification of the targeted region, only the wild-type *tet2* allele can be digested by BsaI. (G) *tet3* TALEN design. Sequences targeted by the left and right TALEN monomers are included in capitals. The Fnu4HI diagnostic restriction site located within the spacer region of the wild-type sequence is underlined. (H) Schematic indicating the 14 base pair deletion in the *tet3*^{mk18} allele. Amino acids encoded by the wild-type sequence are indicated at the top. The Fnu4HI restriction site, which is destroyed in *tet3*^{mk18}, is underlined. (I) Representative gel genotyping larvae for the *tet3*^{mk18} mutation. Following PCR amplification of the targeted region, only the wild-type *tet3* allele can be digested by Fnu4HI.


A
TCGACCCGACCGCACACACcccacccgaatctctcTGGTTTTCTACCAGCACAA
HinfI
tet1
B
P T R I S L V
wt
tet1mk16/mk16
HinfI (GANTC)
4 bp deletion
C
uncut wt tet1mk16/+ tet1mk16/mk16
D
TAGACTTCTGTGCTCATGCtcacagagacctccacAACATGCAAGGGGGCAGTA
BsaI
tet2
E
H A H R D L H N
wt
tet2mk17/mk17
BsaI (GAGACC)
4 bp deletion
F
uncut wt tet2mk17/+ tet2mk17/mk17
G
TGACCGCACTCACCCAagccgcatatccctgGTCTTCTACCAGCACAAGAA
Fnu4HI
tet3
H
S R I S L V F
wt
tet3mk18/mk18
Fnu4HI (GCNGC)
14 bp deletion
I
uncut wt tet3mk18/+ tet3mk18/mk18

WISH

WISH was performed as described (Thisse and Thisse, 2008). For all probes except *gata2b*, 10% dextran sulfate was added to the hybridization buffer.

RNA synthesis and microinjection

The human *TET3* vector used for mRNA production has been previously described (Ko et al., 2013). The human *TET2* ORF corresponding to Genebank: NM_001127208 was amplified from SY5Y cDNA and following sub-cloning, was introduced into the pEF1/V5-His vector (Invitrogen) to allow for *in vitro* transcription. Mutant TET2 (H1382Y, D1384A) was generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). pExpress-1-gata2b was purchased from Transomic Technologies. For *scl-β*, RT-PCR amplified *scl-β* cDNA with sequence corresponding to Genbank: EF488003 was cloned into pCS2+. Sequences of all clones were confirmed by conventional DNA sequencing. Tet1 plasmid was purchased from Addgene. Mutant Tet1 D2018A was obtained from the Panning lab. In all cases, capped RNA was synthesized using mMessage mMachine (Ambion) with SP6 or T7 polymerase as appropriate to the vector. For each experimental condition, mRNA was injected into at least fifty embryos derived from $^{tet2mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses.

5hmC Dot Blot

Genomic DNA was isolated from larvae at 30hpf by phenol-chloroform extraction and ethanol precipitation. Following RNase treatment and denaturation, 2-fold serially diluted DNA was spotted onto nitrocellulose membranes. Cross-linked membranes were incubated with 0.02% methylene blue to validate uniform DNA loading. Membranes were blocked with 5% BSA and incubated with anti-5hmC antibody (1:10,000; Active Motif) followed by a horseradish peroxidase-conjugated antibody (1:15,000; Active Motif). Signal was detected using the ECL Prime Detection Kit (GE).

Time-lapse confocal microscopy

Embryos were anaesthetized with 0.02% tricaine and embedded in 0.5% low-melt agarose. Embryos were scanned using a SP8 confocal microscope (Leicia) at 28.5°C. Confocal z-stacks were acquired every 7 to 10 minutes between 30 and 46 hpf. Approximately 25 planes were collected per time point at a spacing of 3 µm. Data was analyzed using Imaris software and exported in QuickTime. All confocal planes were sequentially analyzed to identify changes in cell morphology consistent with EHT and to identify nuclear fragmentation events.

TP1:GFP imaging and quantification

Mounted samples were scanned using an SP8 confocal microscope (Leica) using 40X water immersion objective at 26 hpf. For each image, approximately 60 planes were captured at a spacing of 0.38um. Data processed using Imaris software and quantified using ImageJ.

Fluorescent activated cell sorting

Zebrafish larvae carrying *kdrl:mCerry* transgene were digested with 0.25% Trypsin-EDTA at 37°C for 20-30 minutes before adding DMEM10 to stop the digestion. Cell pellets were collected after centrifugation at 500g for 10 minutes and subsequently re-suspended in 500ul DMEM. The re-suspended cell solution was filtered by passing through 5ml polystyrene round-bottom tube with cell-strainer cap (12 × 75mm). Cell sorting was performed by the MSKCC Flow Cytometry Core Facility.

RNA isolation and qRT-PCR

Zebrafish larvae were lysed in Trizol and total RNA was isolated by ethanol precipitation. The cDNAs were synthesized using GoScript Reverse Transcription system (Promega, A5000). Quantitative real-time PCR was performed in duplicate using Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659) on the ABI PRISM 7500 Real Time PCR System (Applied Biosystems) using the following parameters: 12 min at 95°C followed by 40 cycles of 20s at 95°C, 30s at 55°C, and 30s at 70°C.

RNA sequencing

Mutant and sibling zebrafish larvae carrying *kdrl:mCerry* were lysed and *mCherry*+ cells were collected for RNA sequencing. On average 8,000 *mCherry*+ cells were harvested from 30 larvae for each sample within the triplicates. Sample cells were re-suspended in Trizol LS reagent for RNA extraction. We set 40 millions reads, 100bp paired ends as the parameters of the RNA sequencing. cDNAs were amplified using SMARTer cDNA synthesis kit (Clontech). The RNA extraction and RNA sequencing were performed by the MSKCC Integrated Genomic Operating Core Facility.

5mC analysis

Genomic DNA was prepared from *kdrl:mCherry* positive FACS sorted cells isolated from pools of roughly 40 mutant or sibling larvae at 28 hpf. DNA was bisulfite converted using the EZ methylation kit (Zymo-Research). PCR products were cloned into pGEM-T Easy Vector System (Promega) and sequenced. Data were analyzed by QUMA (Kumaki et al., 2008).

Data Quantification and Statistical analysis

Quantification data were presented as mean ± SEM. The Student unpaired 2-tailed t-test was used for statistical analysis using Microsoft Excel.

CHAPTER FIVE: REFERENCES

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APPENDIX ONE: Cell Reports Paper

Article

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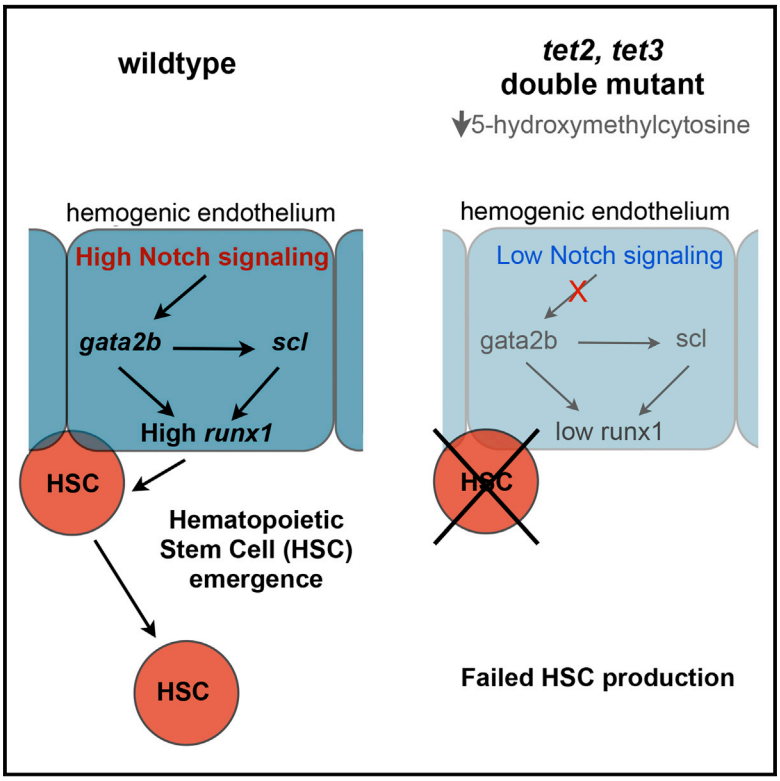
Overlapping Requirements for Tet2 and Tet3 in Normal Development and Hematopoietic Stem Cell Emergence

Graphical Abstract



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In Brief

The Tet proteins comprise a family of dioxygenases that convert 5-methylcytosine to 5-hydroxymethylcytosine. Li et al. identify Tet2 and Tet3 as the major 5-methylcytosine dioxygenases in the zebrafish embryo and uncover an overlapping requirement for Tet2 and Tet3 in hematopoietic stem cell emergence.

Highlights

- Tet2 and Tet3 are the major 5-methylcytosine dioxygenases in the zebrafish embryo
- Tet2 and Tet3 have overlapping requirements in hematopoietic stem cell emergence
- Loss of Tet2/3 compromises expression of the *gata2b/scl/runx1* hematopoietic program
- Notch signaling in the hemogenic endothelium is dependent on Tet2/3

Li et al., 2015, Cell Reports *12*, 1133–1143
August 18, 2015
http://dx.doi.org/10.1016/j.celrep.2015.07.025

Cell Reports
Article

Overlapping Requirements for Tet2 and Tet3 in Normal Development and Hematopoietic Stem Cell Emergence

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http://dx.doi.org/10.1016/j.celrep.2015.07.025

SUMMARY

The Tet family of methylcytosine dioxygenases (Tet1, Tet2, and Tet3) convert 5-methylcytosine to 5-hydroxymethylcytosine. To date, functional overlap among Tet family members has not been examined systematically in the context of embryonic development. To clarify the potential for overlap among Tet enzymes during development, we mutated the zebrafish orthologs of *Tet1*, *Tet2*, and *Tet3* and examined single-, double-, and triple-mutant genotypes. Here, we identify Tet2 and Tet3 as the major 5-methylcytosine dioxygenases in the zebrafish embryo and uncover a combined requirement for Tet2 and Tet3 in hematopoietic stem cell (HSC) emergence. We demonstrate that Notch signaling in the hemogenic endothelium is regulated by Tet2/3 prior to HSC emergence and show that restoring expression of the downstream *gata2b/scl/runx1* transcriptional network can rescue HSCs in *tet2/3* double mutant larvae. Our results reveal essential, overlapping functions for *tet* genes during embryonic development and uncover a requirement for 5hmC in regulating HSC production.

INTRODUCTION

In vertebrate species, the epigenetically modified base 5-methylcytosine (5mC) is associated with transcriptional repression and is essential for normal development (Goll and Bestor, 2005). The mechanisms that establish and maintain 5mC are well defined, but less is known about how 5mC is removed (Wu and Zhang, 2011). The ten-eleven translocation proteins (Tet1, Tet2, and Tet3) comprise a family of 2-oxoglutarate- and Fe(II)-dependent dioxygenases that convert 5mC to 5-hydroxymethylcytosine (5hmC) and its oxidative derivatives 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (He et al., 2011; Tahiliani et al., 2009). Growing evidence suggests that conversion of 5mC to 5hmC and its derivatives can provide a first step toward DNA demethylation through active base excision or passive dilution of oxidized bases (Kohli and Zhang, 2013). Consistent with a role in regulating gene expression through DNA demethylation, 5hmC levels are most abundant in euchromatic regions, including transcription start sites, enhancers, and exons (Pastor et al., 2011; Stroud et al., 2011; Williams et al., 2011).

Individually, homozygous mutation of *Tet1*, *Tet2*, or *Tet3* is compatible with mouse embryonic development, although *Tet3* mutant mice die perinatally (Kohli and Zhang, 2013). Conditional deletion of *Tet3* in oocytes results in delayed demethylation of the paternal genome and increased developmental failure, but viable pups can be recovered (Gu et al., 2011; Inoue et al., 2015). In contrast to single mutants, embryonic stem cells (ESCs) mutated for all three *Tet* genes contribute poorly to chimeras, suggesting that Tet family members have overlapping functions in promoting embryonic development (Dawlaty et al., 2014). *Tet1/2* double-homozygous-mutant mice can survive into adulthood, but other *Tet* mutant combinations have yet to be described (Dawlaty et al., 2013).

Tet regulation appears to be of particular importance in the hematopoietic lineage. *TET1* was first identified as a fusion partner of the mixed lineage leukemia (MLL) gene in acute myeloid leukemia and has essential oncogenic roles in MLL rearranged leukemias (Huang et al., 2013; Lorsbach et al., 2003). Moreover, *Tet1* mutations promote increased self-renewal of progenitor B cells and susceptibility to B cell lymphoma in mice (Cimmino et al., 2015). Mutations in *TET2* are common in human myeloid malignancies, and *Tet2* mutation promotes myeloid transformation in mice and zebrafish (Gjini et al., 2015; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). Consistent with a role in promoting myeloid malignancy, *Tet2* mutation causes increased numbers of hematopoietic progenitor cells in the bone marrow and skewed differentiation toward the myelomonocytic lineage in mice (Ko et al., 2015). Similarly, human cord blood cells

depleted for *TET2* and cells isolated from leukemia patients bearing *TET2* mutations exhibit an increase in myeloid-lineage differentiation at the expense of the erythroid-lineage (Madzo et al., 2014; Pronier et al., 2011). Recently, mutation of *Tet3* was shown to cause minor decreases in the absolute number of hematopoietic stem cells (HSCs) in the mouse bone marrow, while numbers of myeloid, erythroid and B lymphoid cells were unaffected (Ko et al., 2015).

While the importance of Tet regulation in the adult hematopoietic system is clear, less is known about requirements for *Tet* genes during early stages of hematopoietic development. Reports using antisense morpholino depletion in zebrafish and shRNA depletion in human ESCs have implicated Tet2 in the regulation of primitive hematopoiesis; but, these results are at odds with the normal primitive hematopoiesis observed in *Tet2* mutant mice and zebrafish (Ge et al., 2014; Gjini et al., 2015; Ko et al., 2015; Langlois et al., 2014). The de novo generation of HSCs during the definitive wave of hematopoiesis also appears normal in *Tet2* mutant mouse and zebrafish embryos; however, the potential for additional Tet enzymes to contribute to HSC emergence during embryonic development has not been experimentally addressed in mutant animals (Gjini et al., 2015; Ko et al., 2015).

The zebrafish genome encodes single well-conserved orthologs of *Tet1*, *Tet2*, and *Tet3* (Almeida et al., 2012). To define the requirements for these genes during development, we generated stable lines carrying mutations in each of the zebrafish *tet* orthologs and derived single-, double-, and triple-homozygous-mutant larvae representing all genetic combinations. In this study, we demonstrate that Tet2 and Tet3 are the major 5mC dioxygenases in the zebrafish embryo and that they have overlapping functions in promoting normal development. In addition, we describe a combined requirement for *tet2* and *tet3* in the de novo generation of HSCs in the embryo, and we demonstrate the importance of *tet2/3* for Notch signaling and downstream expression of the *gata2b/scl/runx1* transcriptional program in the hemogenic endothelium. Our results provide a comprehensive analysis of *tet* mutants in the developing zebrafish embryo and identify requirements for Tet regulation in the early function of the hemogenic endothelium. These results underscore the importance of epigenetic regulation for the generation of HSCs and identify regulation of 5hmC as an additional variable to be considered in therapeutic applications such as the in vitro differentiation of HSCs from pluripotent precursors.

RESULTS

TALEN-Induced Mutations Reveal Overlapping Requirements for tet2 and tet3 in the Zebrafish Embryo

To systematically define requirements for 5hmC during development, we introduced mutations into the zebrafish orthologs of *Tet1*, *Tet2*, and *Tet3* using TAL effector nucleases (TALENs) (Li et al., 2011a; Sander et al., 2011). RNAs encoding TALENs that targeted each gene were separately injected into one-cell-stage embryos, and we recovered individuals harboring germline transmissible mutations in each of the three genes. The recovered $tet2^{mk17}$ allele deletes 4 bp in exon 8. This deletion results in a frameshift, causing early termination one amino acid 3′ of an essential iron-binding residue (Figures 1A and 1B; Figures S1D–S1F). The $tet1^{mk16}$ and $tet3^{mk18}$ alleles harbor 4-bp and 14-bp deletions, respectively, in the last coding exon. In addition to causing frameshift and premature termination, these deletions eliminate sequence encoding a C-terminal arginine residue that is required for 2-oxoglutarate binding (Hu et al., 2013) (Figures 1A and 1B; Figures S1A–S1C and S1G–S1I). Loss of specific residues involved in cofactor binding and catalysis is predicted to similarly compromise the dioxygenase activity of all three enzymes.

Modest (<3-fold) reductions in total 5hmC were observed in larvae that were homozygous mutant for *tet1*, *tet2*, or *tet3* (Figure 1C). More considerable reductions in 5hmC were observed in double mutants, with the $tet2^{mk17/mk17}$, $tet3^{mk18/mk18}$ double-mutant ($tet2/3^{DM}$) combination producing the most dramatic decrease (>30-fold) (Figure 1C). 5hmC levels were further reduced in *tet1/2/3* triple-mutant ($tet1/2/3^{TM}$) larvae, indicating that all three mutated genes encode proteins that are compromised for catalytic activity (Figure 1D). The enhanced loss of 5hmC in $tet2/3^{DM}$ larvae compared to other double-mutant combinations argues that Tet2 and Tet3 are the predominant 5mC oxidases in the zebrafish embryo and that they function redundantly to promote the formation of 5hmC during development.

Zebrafish that were homozygous for mutations in *tet1*, *tet2*, or *tet3* were viable to adulthood, as were *tet1/2* and *tet1/3* double-homozygous mutants. In contrast, combined mutation of *tet2* and *tet3* was not compatible with survival beyond the larval period. The $tet2/3^{DM}$ larvae were morphologically indistinguishable from wild-type controls during the first 24 hr postfertilization (24 hpf), but subtle abnormalities in brain development emerged on the second day postfertilization (2 dpf). By 36 hpf, smaller eyes, abnormal brain morphology, altered pigmentation, and a modest curvature of the trunk were apparent in 25% of larvae derived from intercrosses between $tet2^{mk17/mk17}$, $tet3^{mk18/+}$ adults (Figure 1E). Genotyping (n = 20) confirmed that the morphologically abnormal embryos all carried homozygous mutations in both *tet2* and *tet3*, demonstrating a combined requirement for the two genes in zebrafish development. Despite the additional reduction in 5hmC, $tet1/2/3^{TM}$ larvae were morphologically indistinguishable from *tet2/3* double mutants at all stages examined (Figure 1F; data not shown).

Overlapping Requirements for Tet2 and Tet3 in Definitive, but Not Primitive, Hematopoiesis

Given the combinatorial effects of *tet2* and *tet3* mutation on overall development, as well as the known roles of Tets in later stages of hematopoiesis, we tested whether *tet2* and *tet3* had overlapping roles in regulating hematopoiesis during embryonic development. Development of non-HSC-derived primitive erythroid and myeloid cells was normal in $tet2/3^{DM}$ larvae and $tet2^{mk17/mk17}$ siblings as assessed by expression of *gata1*, *scl*, and *pu.1* at 25 hpf by whole-mount in situ hybridization (WISH) (Figures 2A–2I). In contrast, expression of HSC-dependent definitive blood cell markers was dramatically reduced in $tet2/3^{DM}$ larvae compared to stage-matched wild-type controls. While mature *rag1*-positive T cells were readily observed in the

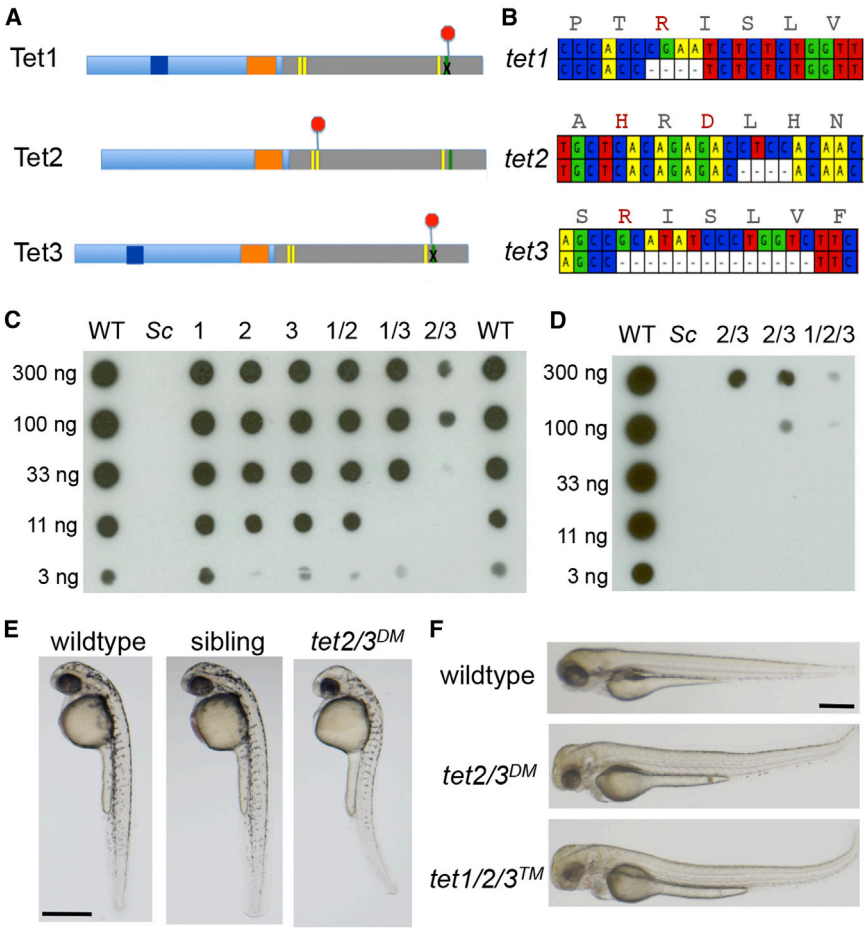


Figure 1. Mutation of Zebrafish *tet1*, *tet2*, and *tet3*

(A) Schematic illustrating early termination caused by TALEN mutations in zebrafish *tet1*, *tet2*, and *tet3*. Red octagons indicate the position of early termination signals. Yellow bars indicate conserved iron binding residues, and the green bar indicates the arginine required for 2-oxoglutarate binding.

(B) Schematic depicting deleted bases in zebrafish *tet1*, *tet2* and *tet3*. Corresponding amino acid sequences for the wild-type allele are included, with residues required for cofactor binding or catalysis indicated in red.

(C) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf. Numbers indicate the mutated *tet* gene(s) in each sample. *Sc* indicates DNA isolated from *Saccharomyces cerevisiae*. Horizontal rows depict 3-fold serial dilutions of DNA.

(D) Dot blot for 5hmC on genomic DNA isolated from larvae at 5 dpf including DNA isolated from *tet1/2/3*^{TM} larvae.

(E) Lateral views of a representative wild-type larva, a *tet2/3*^{DM} larva and a sibling larva derived from a *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercross at 36 hpf.

(F) Lateral views of a representative wild-type larva, a *tet2/3*^{DM} larva and *tet1/2/3*^{TM} larva at 3 dpf.

All scale bars indicate 500 μM. See also Figure S1.

thymus of wild-type larvae at 5 dpf, *rag1*-positive cells were absent from the corresponding region of *tet2/3*^{DM} larvae (Figure 3A and 3B). Similarly, the *Tg(lysC:GFP)* transgenic line, which labels definitive granulocytes (Hall et al., 2007), produced a clear fluorescent signal in blood from wild-type larvae at 6 dpf, but fluorescent cells were not detected in *tet2/3*^{DM} larvae at this stage (Figures 3C and 3D).

The loss of differentiated definitive blood cells in *tet2/3*^{DM} larvae can be attributed to a defect in HSC development. During normal embryonic development, HSCs emerge from the hemogenic endothelium in the ventral wall of the dorsal aorta (DA). In *tet2/3*^{DM} larvae, we found that expression of the HSC-associated genes *runx1* and c-*myb* was reduced in the DA at 36 hpf, whereas *runx1* expression in *tet2* and *tet3* single homozygous mutant larvae was indistinguishable from wild-type (Figures 3E–3M). Moreover, in *tet2/3*^{DM} larvae, *c-myb* positive hematopoietic stem and progenitor cells (HSPCs) could not be detected in the caudal hematopoietic tissue (CHT) niche, a secondary, transient site for HSC amplification (Figures S2A–S2F). Importantly, *runx1* expression was rescued by injecting mRNA encoding either human TET2 or TET3 into one-cell-stage embryos derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses (Figure 3N). Injection of mRNA encoding a catalytically dead version of TET2 failed to rescue wild-type levels of *runx1* expression, directly implicating the 5mC dioxygenase activity of TET2 in regulating HSC development (Figure 3N).

Normal vascular development, arterial specification, and blood-flow-induced nitric oxide (NO) signaling are known prerequisites for HSC development (Adamo et al., 2009; Jagannathan-Bogdan and Zon, 2013; North et al., 2009). WISH for the vascular markers *kdrl* and *cdh5* and the arterial marker *efnb2a* revealed similar expression in wild-type and *tet2/3*^{DM} larvae, demonstrating the presence of an overtly intact vasculature (Figures 4A–4F). Visual inspection of *tet2/3*^{DM} larvae by bright-field microscopy showed that blood flow was grossly normal during the first 2 days of development, and *klf2a*, an immediate early responder to blood flow, was expressed at similar levels in wild-type and *tet2/3*^{DM} larvae (Figures 4G and 4H). Moreover, while exposure to the NO agonist S-nitroso-N-acetyl-penicillamine (SNAP) was sufficient to rescue *runx1* expression in *silent heart* (*sih*) morpholino-injected embryos lacking blood circulation, SNAP exposure was unable to rescue *runx1* expression in *tet2/3*^{DM} larvae (Figures S3A–S3E). Taken together, these results suggest that the HSC defects observed in *tet2/3*^{DM} larvae are not secondary to defects in vascular development or aberrant blood flow.

HSC Emergence Is Compromised in tet2/3 ^{DM} Larvae

Beginning around 32 hpf, nascent HSCs emerge from the ventral aortic endothelium of the zebrafish embryo through a process termed the endothelial to hematopoietic transition (EHT) (Bertrand et al., 2010; Kissa and Herbomel, 2010). To

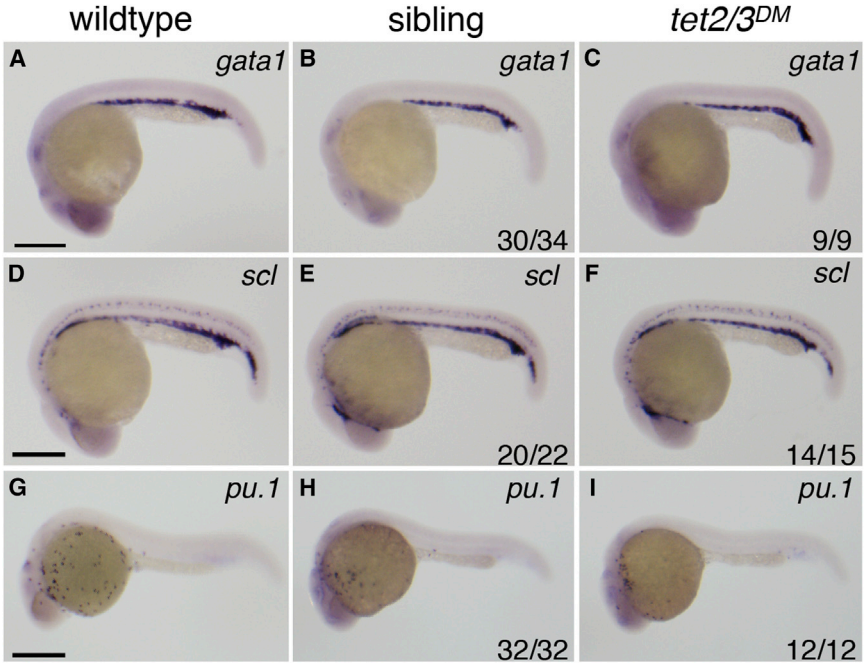


Figure 2. Markers of Primitive Hematopoiesis Are Similarly Expressed in Wild-Type, *tet2*$^{mk17/mk17}$, and *tet2/3*^{DM} Larvae

(A–I) Representative lateral views of wild-type larvae compared to *tet2/3*^{DM} and *tet2*$^{mk17/mk17}$ sibling larvae derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses.

(A–C) WISH for the primitive erythroid marker *gata1* at 25 hpf.

(D–F) WISH for the primitive erythroid marker *scl* at 25 hpf.

(G–I) WISH for the primitive myeloid marker *pu.1* at 25 hpf. Numbers in the lower right hand corner indicate the fraction of embryos exhibiting WISH labeling similar to the representative image.

Scale bars indicate 500 μM.

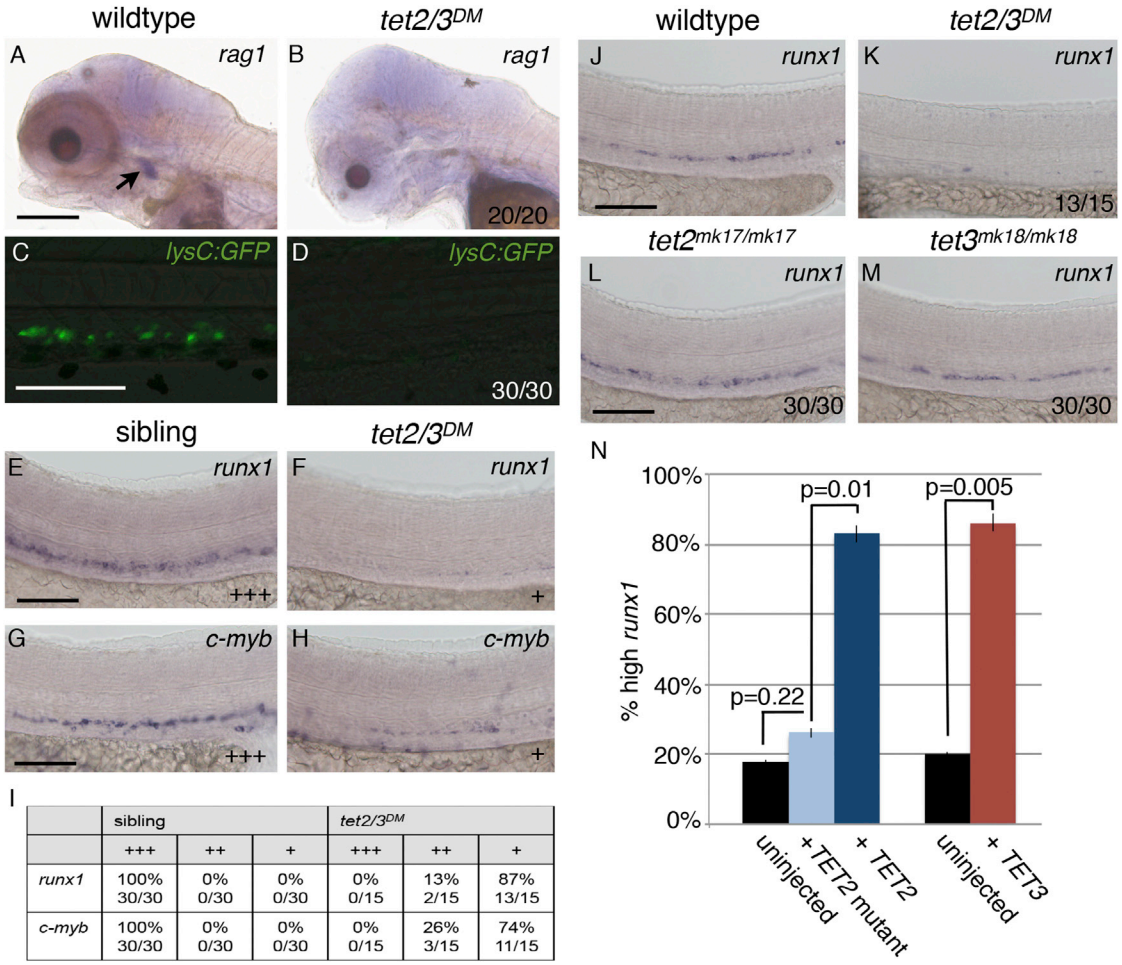
test whether EHT was impacted by mutation of *tet2/3*, the *tet2* and *tet3* mutant alleles were introduced into a *Tg(kdrl:Ras-mCherry)*^{S896}, *Tg(kdrl:H2B-EGFP)*^{mu122} transgenic background (Chi et al., 2008; Kochhan et al., 2013). In this background, membrane mCherry and nuclear GFP are expressed in the vascular endothelium and emergent HSCs, allowing EHT events to be identified based on stereotypical changes in cell morphology (Bertrand et al., 2010; Kissa and Herbomel, 2010). Prior to the onset of EHT, *tet2/3*^{DM} larvae and siblings from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses exhibited similar fluorescent labeling with the two transgenes (Figures 5A and 5B). A defined region of the DA was then monitored in *tet2/3*^{DM} larvae and sibling controls (n = 3 each) between 30 and 46 hpf by time-lapse confocal microscopy. Analysis of image sets revealed a 4-fold reduction in the number of EHT events detected in *tet2/3*^{DM} larvae compared to siblings, indicating that EHT is compromised in the double mutants (average of 3 versus 13 EHT events, p = 0.001; Figures 5C and 5E; Movie S1).

In each time-lapse sequence from *tet2/3*^{DM} larvae, we also observed between one and three cells within the DA undergoing nuclear fragmentation (Figures 5D and 5F; Movie S2). In contrast, fragmentation was never detected in time-lapse sequences from siblings (Figure 5D). The nuclear fragmentation phenotype we observed in *tet2/3*^{DM} larvae is reminiscent of that described for zebrafish embryos depleted for *runx1* by morpholino injection and suggests that a fraction of cells undergoing EHT also die via apoptosis in the absence of *tet2/3* (Kissa and Herbomel, 2010). Elevated levels of TUNEL-positive cells were not observed in the DA or surrounding tissues of fixed *tet2/3*^{DM} larvae (n = 10, data not shown). However, apoptotic events restricted to the few cells undergoing EHT would be difficult to capture in fixed embryos.

Tet2/3 Are Required for Notch Signaling and Expression of Key Hematopoietic Transcription Factors in the Hemogenic Endothelium

The reduction in EHT events in *tet2/3*^{DM} larvae suggested a requirement for Tet2/3 in the function or specification of the hemogenic endothelium, which gives rise to nascent HSCs. The hematopoietic transcription factors *runx1* and *scl* are both expressed in the hemogenic endothelium prior to the initiation of EHT and are required for this process (Kissa and Herbomel, 2010; Zhen et al., 2013). By WISH, we found that expression of both *runx1* and *scl* was reduced in the DA of *tet2/3*^{DM} larvae prior to HSC emergence, indicating that Tet2 and Tet3 are required to promote the hemogenic potential of the vascular endothelium (Figures 6A–6D). Expression of *scl* and *runx1* is controlled in part by the transcription factor Gata2 in mouse, and the zebrafish genome encodes for two Gata2 paralogs that have undergone subfunctionalization (Butko et al., 2015; Gao et al., 2013; Göttgens et al., 2002; Pimanda et al., 2007). Zebrafish *gata2a* is broadly expressed throughout the hematopoietic system and is important for vascular morphogenesis (Zhu et al., 2011). In contrast, zebrafish *gata2b* is specifically detected in the hemogenic endothelium and is required for *runx1* expression within this tissue (Butko et al., 2015). Consistent with the HSC-specific phenotypes observed in *tet2/3*^{DM} larvae, we found that *tet2/3* mutation compromised expression of *gata2b*, while *gata2a* expression was unaffected at similar stages (Figures 6E–6H). Collectively, these observations identify Tet2/3 as essential regulators of the *gata2b/scl/runx1* transcriptional network in the hemogenic endothelium.

To clarify whether disruption of *gata2b/scl/runx1* transcriptional program could account for the HSC defects observed in *tet2/3* double-mutant larvae, we next tested whether reintroducing mRNA encoding *scl* or *gata2b* into *tet2/3*^{DM} embryos could rescue HSC production. In-vitro-transcribed mRNA encoding Scl or Gata2b was injected into one-cell-stage embryos derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses, and *runx1* expression was subsequently examined in the DA of *tet2/3* double mutants by WISH at 36 hpf. Injection of either mRNA was sufficient to



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	sibling			*tet2/3*^{DM}		
	+++	++	+	+++	++	+
runx1	100% 30/30	0% 0/30	0% 0/30	0% 0/15	13% 2/15	87% 13/15
c-myb	100% 30/30	0% 0/30	0% 0/30	0% 0/15	26% 3/15	74% 11/15

Figure 3. *tet2* and *tet3* Have Overlapping Functions in HSC Development

(A and B) WISH for *rag1* at 5 dpf. Arrow indicates thymic T cells in wild-type larvae.

(C and D) GFP labeled macrophages and neutrophils in 6 dpf larvae carrying the *Tg(lysC:GFP)* transgene.

(E and F) WISH for the HSC marker *runx1* in the DA at 36 hpf.

(G and H) WISH for the HSC marker *c-myb* in the DA at 36 hpf.

(I) Number of sibling and *tet2/3*^{DM} embryos with wild-type (+++), reduced (++), or nearly absent (+) *runx1* labeling in the DA. Numbers are representative of three independent crosses.

(J–M) WISH for *runx1* in the DA at 32 hpf.

(N) Graph indicating the percent of *tet2/3*^{DM} larvae exhibiting high *runx1* staining in the DA in uninjected controls or following injection with 100 pg mRNA encoding TET2, TET2 H1382Y.D1384A (TET2 mutant), or TET3. Numerical data are presented as the mean ± SEM.

Numbers in the lower right corner of images indicate the fraction of larvae with WISH labeling similar to the representative image. All scale bars indicate 100 μM. See also Figure S2.

rescue wild-type levels of *runx1* expression in the DA of *tet2/3*^{DM} larvae, identifying the *gata2b/scl/runx1* network as the primary hematopoietic program regulated by Tet2/3 during HSC emergence (*scl*: p = 0.0004, *gata2b*: p = 0.0008, Figures 6I–6M).

Analysis of genomic DNA from sorted vascular endothelial cells revealed that the promoters of *gata2b*, *runx1*, and *scl* were similarly unmethylated in wild-type and *tet2/3*^{DM} larvae, suggesting that Tet2/3 do not directly regulate expression of these genes via promoter demethylation (Figure S4). This observation raised the possibility that Tet2/3 act upstream of *gata2b* in regulating HSC emergence. Notch signaling is required for the expression of *gata2b* in the zebrafish hemogenic endothelium, and Gata2 is a direct target of Notch signaling in the mouse dorsal aorta, making this pathway a strong candidate for Tet2/3 regulation during HSC emergence (Butko et al., 2015; Robert-Moreno et al., 2005). To test whether mutation of *tet2/3* disrupted Notch signaling in the hemogenic endothelium, we introduced the *tet2* and *tet3* mutant alleles into a Notch reporter line, *Tp1:GFP*, which expresses GFP under the control of tandem Notch responsive elements (Parsons et al., 2009). At the whole-embryo level, wild-type and *tet2/3*^{DM} larvae exhibited similar patterns of GFP expression from the *Tp1:GFP* transgene, suggesting that Notch signaling was not globally compromised by mutation of *tet2/3* (Figures S5A–S5D). At higher resolution, morphologically wild-type embryos carrying

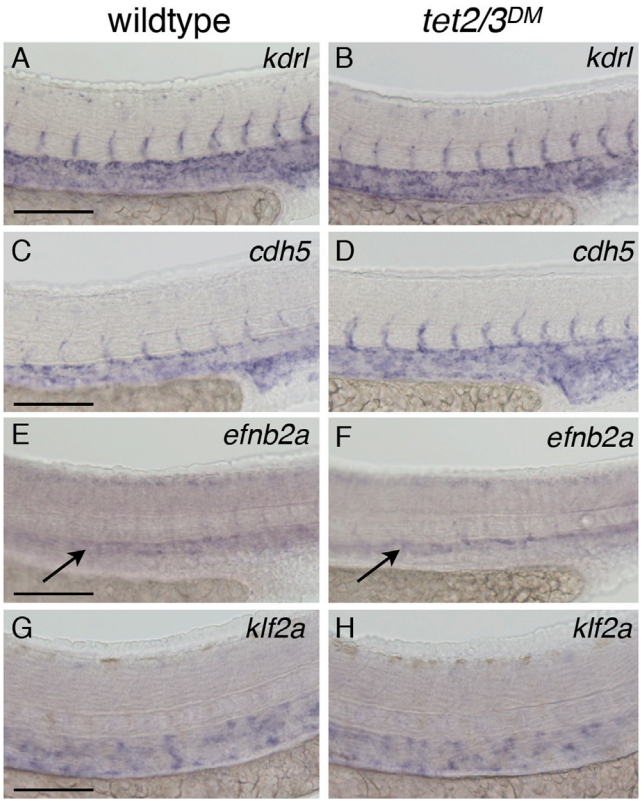

Figure 4. Normal Vasculature and Blood Flow in *tet2/3*^{DM} Larvae
(A and B) WISH for the vascular marker *kdrl at* 31 hpf.
(C and D) WISH for vascular marker *cdh5* at 31 hpf.
(E and F) WISH for the arterial marker *efnb2a* at 31 hpf.
(G and H) WISH for the blood-flow-dependent marker *klf2a* at 36 hpf.
Scale bars indicate 100 μM. See also Figure S3.

the *Tp1:GFP* transgene exhibited the expected strong expression of *Tp1:GFP* along both the dorsal and ventral walls of the DA (Figure 7A). In *tet2/3*^{DM} larvae, *Tp1:GFP* expression on the dorsal side of the DA appeared similar to wild-type; however, expression along the ventral wall appeared weaker and discontinuous (n = 10/11; Figure 7B). Quantification of mean GFP fluorescent intensity in each region revealed a 3-fold reduction in the ratio of GFP fluorescence in the ventral DA compared to the dorsal DA in *tet2/3* double mutants ($p = 0.0002$; Figure 7C). This specific reduction in GFP in the ventral DA reveals a requirement for Tet2/3 in regulating Notch signaling in the hemogenic endothelium and provides a mechanistic explanation for the downregulation of *gata2b* observed in *tet2/3*^{DM} larvae. Taken together these results uncover a requirement for Tet2/3 in the early function of the hemogenic endothelium and identify Notch signaling and the downstream expression of the *gata2b/scl/runx1* transcriptional network as key targets of Tet2/3 regulation during HSC emergence.

DISCUSSION

Our systematic analysis of *tet* mutant phenotypes revealed Tet2 and Tet3 to be the major 5mC dioxygenases in the zebrafish embryo. To date, a number of studies in mouse have focused on *Tet1* and *Tet2*, likely due to the fact that these are the only *Tet* orthologs expressed in ESCs (Wu and Zhang, 2011). Nonetheless, *Tet3* is upregulated upon differentiation of ESCs and is highly expressed in many differentiated primary tissues (Dawlaty et al., 2013; Li et al., 2015). Our analysis of zebrafish *tet* mutants, combined with the survival of *Tet1/2* mutant mice and the poor differentiation capacity of *Tet1/2/3* mutant ESCs, suggest that *Tet2* and *Tet3* may also have important overlapping requirements in promoting mammalian development (Dawlaty et al., 2013, 2014). Intriguingly, while at least *Tet3* is maternally deposited in mouse, few, if any, *tet* transcripts are detected in RNA-sequencing data from two-cell-stage zebrafish embryos, and 5hmC is not detected in the zebrafish embryo by immunofluorescence until the bud stage (Almeida et al., 2012; Gu et al., 2011). These observations imply that mRNAs encoding the Tet enzymes are not maternally deposited in zebrafish and suggest that, in contrast to mammals, the zebrafish genome does not contain significant amounts of 5hmC prior to segmentation. Zebrafish do not undergo the same Tet-dependent erasure and reestablishment of global 5mC patterns observed during mammalian preimplantation development, providing one potential explanation for this distinction (Jiang et al., 2013; Potok et al., 2013). The lack of maternal deposition and limited dependency on Tet enzymes during the first 24 hpf make zebrafish a powerful system for examining Tet requirements in later developmental processes, including those associated with tissue specific development and differentiation.

In the current study, we examine requirements for Tet2/3 during embryonic stages of hematopoietic development. Defects in primitive hematopoiesis following antisense morpholino depletion of *tet2* in zebrafish and impaired differentiation of primitive embryonic/yolk sac progenitors following small hairpin RNA (shRNA) depletion of *TET2* in human ESCs have been reported (Ge et al., 2014; Langlois et al., 2014). However, these results are difficult to reconcile with the normal primitive hematopoiesis observed in published mouse and zebrafish models of *Tet2* mutation (Gjini et al., 2015; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron et al., 2011; Shide et al., 2012; Solary et al., 2014). While it is difficult to definitively address the discrepancies between these studies, both shRNA and antisense morpholino technologies can be susceptible to off-target effects (Kok et al., 2015; Scherer and Rossi, 2003). Similar to other published studies of *Tet2* mutants, we find that primitive hematopoiesis proceeds normally in the *tet2* homozygous-mutant zebrafish generated by our laboratory, and we demonstrate that combinatorial elimination of Tet2 and Tet3 catalytic functions does not further impact primitive hematopoiesis. It is important to note that because the truncation mutations used in this study leave N-terminal coding sequence intact, we cannot rule out the possibility that Tet2/3 have combinatorial dioxygenase independent functions in regulating primitive hematopoiesis.

The earliest stages of definitive hematopoiesis also appear unaffected in *Tet2* single-mutant mice and zebrafish, although diminished expression of *c-myb*, but not *runx1*, was observed in the DA of *tet2* morpholino-injected zebrafish embryos (Ge et al., 2014; Gjini et al., 2015; Ko et al., 2011; Kunimoto et al., 2012; Li et al., 2011b; Moran-Crusio et al., 2011; Quivoron

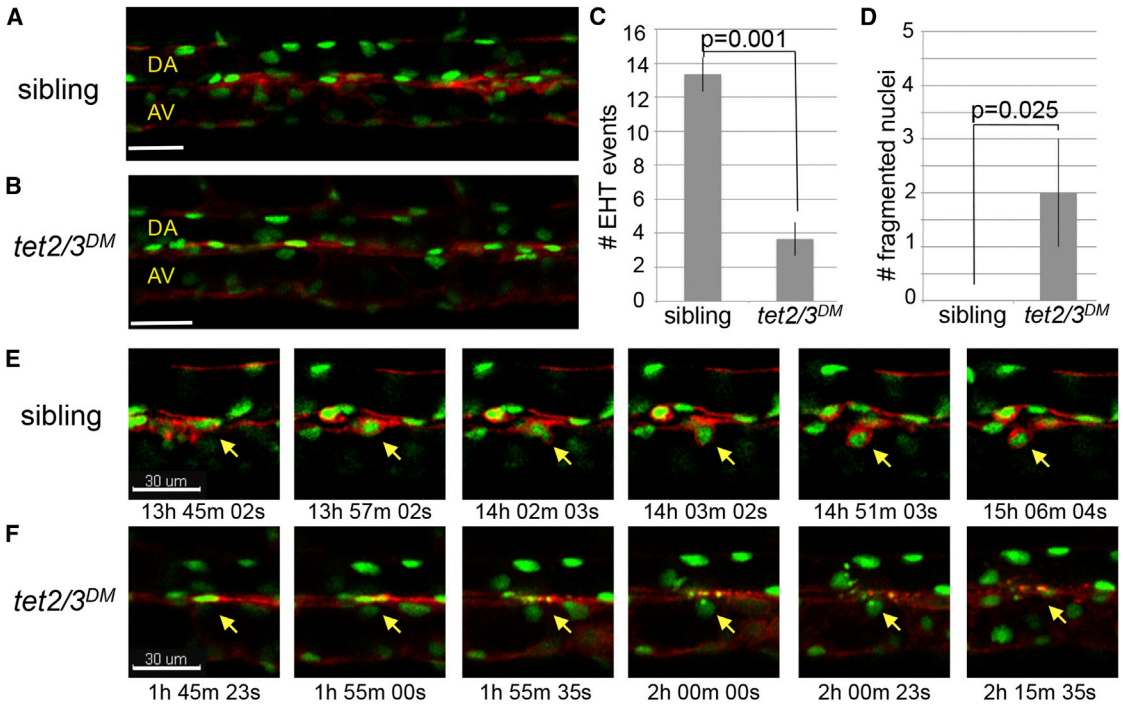

Figure 5. Tet2/3 Are Required for HSC Emergence through the Endothelial to Hematopoietic Transition

(A and B) Merged images depicting GFP and mCherry labeling of the vasculature in *Tg(kdrl:Ras-mCherry)*, *Tg(kdrl:H2B-EGFP)* transgenic larvae at 30 hpf.

(C) Number of EHT events detected between 30 and 46 hpf in *tet2/3*^{DM} larvae and siblings. Numerical data are presented as the mean ± SEM.

(D) Number of fragmented nuclei observed in the DA of *tet2/3*^{DM} larvae and their siblings between 30 and 46 hpf. Numerical data are presented as the mean ± SEM.

(E) Sequences from Movie S1 documenting the stepwise emergence of an HSC from the DA of a sibling larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell undergoing EHT.

(F) Sequences from Movie S2 documenting a cell undergoing nuclear fragmentation in the DA of a *tet2/3*^{DM} larva. For each time point, merged GFP and mCherry images are shown. The yellow arrow indicates the cell with nuclear fragmentation.

All scale bars indicate 30 μM. See also Movies S1 and S2.

et al., 2011; Shide et al., 2012; Solary et al., 2014). In genetic models, loss of *Tet2* eventually leads to an expansion of hematopoietic progenitor cells in the bone marrow and skewed myeloid differentiation (Gjini et al., 2015; Ko et al., 2011; Li et al., 2011b; Moran-Crusio et al., 2011). However, the long latency that precedes these phenotypes suggests secondary somatic mutations may be a contributing factor. In contrast to these later hematopoietic phenotypes, we find that combined mutation of *tet2/3* causes an early loss of definitive blood cells, resulting from compromised HSC production. Importantly, abnormalities in HSC development occurred in embryos that were morphologically quite normal and had normal expression of vascular markers. The relatively normal development of *tet2/3*^{DM} larvae supports a specific role for *tet2* and *tet3* in regulating transcription of the embryonic HSC developmental program rather than a more generalized role in regulating global transcription.

Compared to *tet1*, both *tet2* and *tet3* transcripts are relatively enriched in the DA of the developing zebrafish embryo (Ge et al., 2014). This expression pattern provides a potential explanation for the specific overlapping Tet2/3 requirements in HSC production. Analysis of double-mutant larvae revealed a combined requirement for *tet2/3* in regulating Notch signaling in the hemogenic endothelium, suggesting a role for 5hmC in the specification or early function of this tissue. Enrichment of 5hmC has been reported at Notch receptor and ligand genes in other tissues, but the functional significance of these changes has not been determined (Terragni et al., 2014). Intriguingly, we find Notch signaling to be relatively intact in other tissues of *tet2/3*^{DM} larvae, indicating that Tet2/3 are likely be involved in fine-tuning the activation of this pathway in select cell types. Notch signaling is essential for HSC development in vertebrates and has been implicated in both specification of the dorsal aorta and downstream HSC production (Jagannathan-Bogdan and Zon, 2013; Robert-Moreno et al., 2005). Tet2/3 appear to be dispensable for Notch regulation of arterial specification, as we observe normal expression of the arterial marker *ephrinb2* in *tet2/3*^{DM} larvae. Instead, disruption in Notch signaling in the hemogenic endothelium favors a select requirement for Tet2/3 in the regulation of HSC specification. This model is consistent with the downstream disruption of the *gata2b/scl/runx1* transcriptional network observed in *tet2/3*^{DM} larvae and our observation that reintroducing *scl* or *gata2b* mRNA can rescue HSC production in double mutants. Notably, Notch regulation is dispensable for *scl* expression during the primitive wave of hematopoiesis, which is consistent with the normal *scl* expression observed in *tet2/3*^{DM} primitive erythrocytes (Burns et al., 2005; Kim et al., 2013).

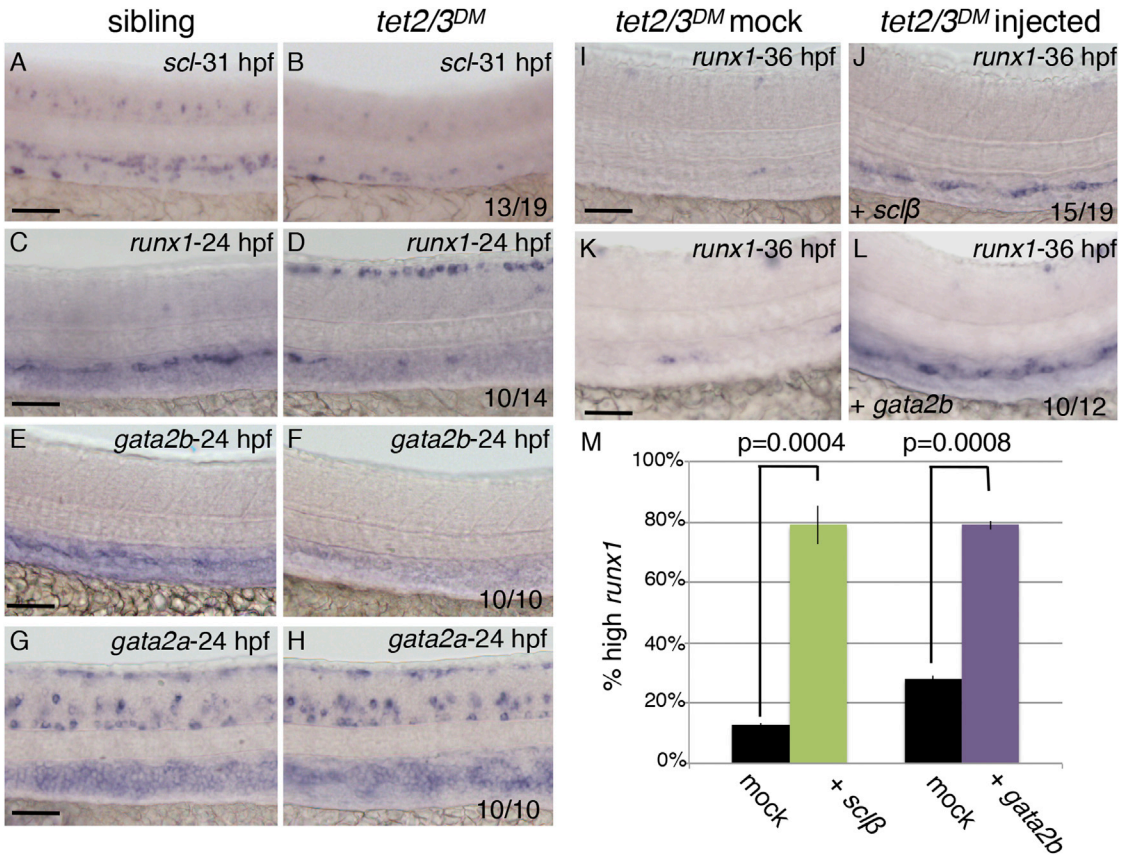

Figure 6. Tet2/3 Regulate Expression of the *gata2b/scl/runx1* Transcriptional Network in the Hemogenic Endothelium

(A and B) WISH for *scl* at 31 hpf.

(C and D) WISH for *runx1* at 24 hpf.

(E and F) WISH for *gata2b* at 24 hpf.

(G and H) WISH for *gata2a* at 24 hpf.

(I and J) WISH for *runx1* in the DA of mock-injected and *sclβ* mRNA-injected *tet2/3*^{DM} embryos at 36 hpf.

(K and L) WISH for *runx1* in the DA of mock-injected and *gata2b* mRNA-injected sibling embryos at 36 hpf.

(M) Percent of *tet2/3*^{DM} embryos with high *runx1* expression following mock injection or injection with mRNA encoding Sclβ (50 pg) or Gata2b (200 pg). Numerical data are presented as the mean ± SEM.

Numbers in the lower right corner of images indicate the fraction of larvae with WISH labeling similar to the representative image. All scale bars indicate 50 μM. See also Figure S4.

Collectively, these results uncover a requirement for Tet regulation of 5hmC in the early function of the zebrafish hemogenic endothelium. A deeper understanding of how HSC generation is regulated in vivo is expected to facilitate the in vitro production of HSCs for therapeutic purposes. Importantly, our results identify regulation of 5hmC as an additional variable to be considered in the optimization of protocols for HSC differentiation from pluripotent progenitors. This observation may be of particular relevance given recent data highlighting the impact of cell culture conditions on global 5hmC levels (Blaschke et al., 2013; Nestor et al., 2015; Yin et al., 2013).

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish maintenance and breeding were conducted under full animal use and care guidelines with approval by the institutional animal care and use committee. Zebrafish were raised under standard conditions at 28°C.

TALEN Mutagenesis

TALEN sequences were selected using Targeter 2.0 software (Doyle et al., 2012). TAL repeat assembly was achieved using the Golden Gate assembly method, and assembled repeats were integrated into the GoldyTALEN scaffold (Bedell et al., 2012; Cermak et al., 2011). Assembled vectors served as templates for in vitro mRNA transcription using the T3 mMessage mMachine kit (Ambion) according to manufacturer's instructions. 50–100 pg mRNA was injected into wild-type embryos at the one-cell stage. Details of mutation recovery and genotyping can be found in Supplemental Experimental Procedures.

5hmC Dot Blot

Genomic DNA was isolated from larvae at 5 dpf by phenol-chloroform extraction and ethanol precipitation. Following RNase treatment and denaturation, serially diluted DNA was spotted onto nitrocellulose membranes. Cross-linked membranes were incubated with 0.02% methylene blue to validate uniform DNA loading. Membranes were blocked with 5% BSA and incubated with anti-5hmC antibody (1:10,000; Active Motif) followed by a horseradish peroxidase-conjugated antibody (1:15,000; Active Motif). Signal was detected using the ECL Prime Detection Kit (GE).

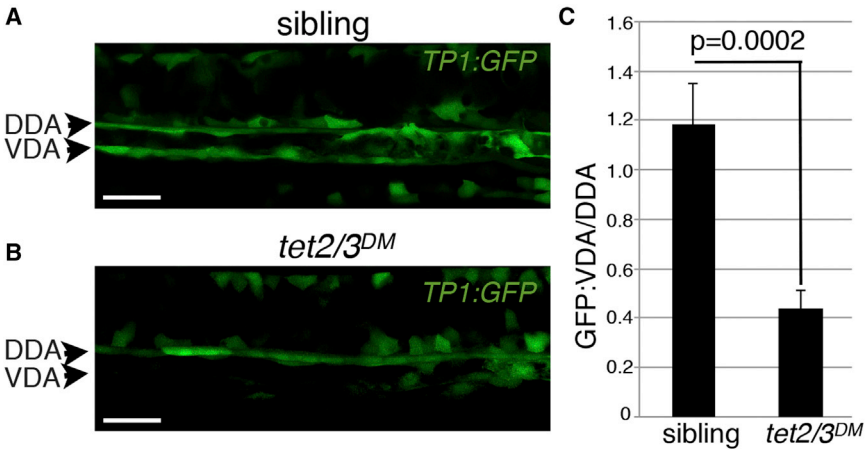

Figure 7. Tet2/3 Are Required for Notch Signaling in the Hemogenic Endothelium

(A and B) Confocal images of *Tp1:GFP* expression in the dorsal aorta of sibling and *tet2/3*^{DM} larvae at 28 hpf. DDA indicates the dorsal wall of the dorsal aorta. VDA indicates the ventral wall of the dorsal aorta.

(C) Ratio of GFP fluorescence intensity in the VDA/DAA in sibling and *tet2/3*^{DM} larvae (n = 11 per genotype). Numerical data are presented as the mean ± SEM. Scale bars indicate 100 μM.

WISH

WISH was performed as described previously (Thisse and Thisse, 2008). For all probes except *gata2b*, 10% dextran sulfate was added to the hybridization buffer.

RNA Synthesis and Microinjection

The human *TET3* vector used for mRNA production has been previously described (Ko et al., 2013). The human *TET2* ORF corresponding to GenBank: NM_001127208 was amplified from cDNA made from SH-SY5Y neuroblastoma cells. Following sub-cloning, the *TET2* ORF was introduced into the pEF1/V5-His vector (Invitrogen) to allow for in vitro transcription. Mutant TET2 (H1382Y, D1384A) was generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). pExpress-1-gata2b was purchased from Transomic Technologies. For *scl-β*, RT-PCR-amplified *scl-β* cDNA with sequence corresponding to GenBank: EF488003 was cloned into pCS2+. Sequences of all clones were confirmed by conventional DNA sequencing. In all cases, capped RNA was synthesized using mMessage mMachine (Ambion) with Sp6 or T7 polymerase as appropriate to the vector. For each experimental condition, mRNA was injected into at least 50 embryos derived from *tet2*$^{mk17/mk17}$, *tet3*$^{mk18/+}$ intercrosses.

Time-Lapse Confocal Microscopy

Embryos were anesthetized with 0.02% tricaine and embedded in 0.5% low-melt agarose. Embryos were scanned using a SP8 confocal microscope (Leica) at 28.5°C. Confocal z stacks were acquired every 7–10 min between 30 and 46 hpf. Approximately 25 planes were collected per time point at a spacing of 3 μm. Data were analyzed using Imaris software and exported in QuickTime. All confocal planes were sequentially analyzed to identify changes in cell morphology consistent with EHT and to identify nuclear fragmentation events.

Tp1:GFP Imaging and Quantification

Mounted samples were scanned using an SP8 confocal microscope (Leica) using 40× water-immersion objective at 26 hpf. For each image, ~60 planes were captured at a spacing of 0.38 μm. Data were processed using Imaris software and quantified using ImageJ.

Statistical Analysis

The Student unpaired 2-tailed t test was used for statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.025.

ACKNOWLEDGMENTS

This work was funded by a Louis V. Gerstner, Jr. Young Investigators Award to M.G.G., grant #2014-010 from the Tri-Institutional Stem Cell Initiative funded by The Starr Foundation to M.G.G. and T.E., and NCI grant P30 CA008748 to MSKCC. We thank the MSKCC Molecular Cytology and Flow Cytometry cores for technical support.

Received: March 6, 2015
Revised: June 19, 2015
Accepted: July 10, 2015
Published: August 6, 2015

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APPENDIX TWO: Methods Paper

CHAPTER

Epigenetic regulation of hematopoietic stem cell development

22

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CHAPTER OUTLINE

Abstract

Hematopoietic stem cells (HSCs) are multipotent self-renewing precursors with the capacity to differentiate into all adult blood cell lineages. HSC development is a highly orchestrated process regulated by multiple transcription factors and signaling pathways.

Methods in Cell Biology, Volume 135, ISSN 0091-679X, http://dx.doi.org/10.1016/bs.mcb.2016.01.010

Emerging evidence suggests that epigenetic regulation is an additional essential component of HSC development. Powerful genetic and imaging approaches, combined with conservation of mammalian programs, have made zebrafish a prominent model for the study of HSC production. This chapter summarizes approaches that have been used to identify epigenetic regulators of HSC development in zebrafish and highlights additional strategies that are likely to facilitate progress in this promising field.

INTRODUCTION

Hematopoietic stem cells (HSCs) are self-renewing precursors from which all adult blood cell types are derived. The production of HSCs during embryonic development is essential for the establishment of the adult blood system (Orkin & Zon, 2008). Transplantation of HSCs derived from bone marrow or cord blood is a true stem cell therapy, used to restore the hematopoietic system in patients with diseased or defective bone marrow. This important therapeutic application has raised significant interest in understanding the mechanisms that regulate the de novo production and maintenance of HSCs in vivo.

The acquisition of cellular identity relies on a combination of genetic and epigenetic information. Genetic information encodes the transcription factors and effector genes that drive progenitor fate and differentiation. In contrast, epigenetic information shapes the accessibility of transcription factors to DNA, providing a heritable chromatin landscape that can direct or reinforce cell type−specific transcription programs. Studies in mice, cell culture, and zebrafish have identified a number of transcription factors and signaling pathways that are important for HSC development (Jagannathan-Bogdan & Zon, 2013). However, much less is known about how epigenetic regulation contributes to the production and maintenance of HSCs in the developing embryo. Zebrafish are well suited to addressing this topic. In this chapter, we provide an overview of approaches that have been used to identify epigenetic regulators of HSC development in zebrafish and we discuss additional strategies that may accelerate progress in this field.

1. MECHANISMS OF EPIGENETIC REGULATION

Epigenetic mechanisms control the accessibility of transcription factors to DNA through the manipulation of chromatin structure. A principle component of chromatin is the nucleosome, which consists of a segment of DNA wound around a core histone octamer. Covalent modifications of DNA and histone tails provide two mechanisms to regulate chromatin compaction; these modifications contribute to a heritable landscape that can influence recruitment of transcriptional machinery through short- and long-range interactions at *cis*-regulatory regions.

1.1 5-METHYLCYTOSINE

In vertebrate genomes, DNA can be modified through methylation of cytosine residues (5-methylcytosine, 5mC). DNA methylation is associated with transcriptional repression and is required for normal development (Goll & Bestor, 2005). The methylation reaction is executed by two groups of DNA methyltransferases: the maintenance methyltransferase, Dnmt1, and the de novo methyltransferases of the Dnmt3 family. De novo methyltransferases methylate unmodified DNA to establish methylation patterns. During maintenance of DNA methylation, the cofactor Uhrf1 recruits Dnmt1 to hemimethylated CpG dinucleotides at the replication fork, allowing faithful propagation of methylation patterns to the newly synthesized DNA strand (Bostick et al., 2007; Sharif et al., 2007). The zebrafish genome encodes single well-conserved orthologs of Dnmt1 and Uhrf1. During the first 2 days of development, *uhrf1*- and *dnmt1*-mutant zebrafish larvae appear overtly normal and genome-wide methylation patterns are largely unaffected, perhaps due to significant maternal deposition of mRNA encoding Dnmt1 and Uhrf1 (Chu et al., 2012; Martin, Laforest, Akimenko, & Ekker, 1999). However, homozygous mutations in either gene result in reduced genome-wide levels of DNA methylation and larval lethality by 7 days post fertilization (dpf) (Anderson et al., 2009; Sadler, Krahn, Gaur, & Ukomadu, 2007; Tittle et al., 2011). Mammalian genomes encode two de novo DNA methyltransferases (Dnmt3A and Dnmt3b), whereas the zebrafish genome encodes six Dnmt3 orthologs (Goll & Halpern, 2011; Shimoda, Yamakoshi, Miyake, & Takeda, 2005; Smith, Dueck, Mhanni, & McGowan, 2005). Mutations in individual zebrafish *dnmt3* genes have not been uncovered in forward genetic screens. It is possible that functional redundancies among these genes have prevented their detection using unbiased approaches.

1.2 5-HYDROXYMETHYLCYTOSINE

While the mechanisms that establish and maintain DNA methylation are well studied, the pathways that regulate removal of 5mC have been more elusive (Wu & Zhang, 2011). In 2009, the Tet proteins (Tet1, Tet2 and Tet3) were identified as a family of cytosine dioxygenases that are capable of converting 5mC to 5-hydroxymethylcytosine (5hmC) and its derivatives 5-formylcytosine and 5-carboxylcytosine (He et al., 2011; Tahiliani et al., 2009). Conversion of 5mC to 5hmC and its derivatives appears to drive DNA demethylation through the active excision or passive dilution of oxidized bases (Wu & Zhang, 2011). Tet-mediated DNA demethylation provides one mechanism for reactivation of genes that have been transcriptionally silenced by 5mC. It is also possible that in some contexts 5hmC may act as a unique DNA modification that imparts distinct epigenetic information on the underlying genome. The zebrafish genome encodes single orthologs of Tet1, Tet2, and Tet3, and combinatorial mutation of these three genes reduces 5hmC in total genomic DNA to levels below the threshold for antibody detection (Almeida et al., 2012; Li et al., 2015).

1.3 HISTONE MODIFICATION

The tails of individual histones within nucleosome are also subject to a variety of covalent modifications that regulate chromatin compaction (Bannister & Kouzarides, 2011). Histone tails are well conserved between zebrafish and other species and the zebrafish genome encodes orthologs of many histone-modifying enzymes identified in mammals. Among the most well-studied histone tail modifications are histone acetylation and methylation. Histone acetylation is regulated by the antagonistic action of histone acetyltransferases and deacetylaces. Acetylation is generally associated with open chromatin structure and active gene expression, whereas histone deacetylation is correlated with gene repression (Eberharter & Becker, 2002). Histone methylation represents a more complex level of regulation as residues can be mono-, di-, or trimethylated and methylation can facilitate gene activation or repression depending on which histone tail residues are modified and to what degree. Polycomb and trithorax complexes represent two groups of proteins that are important for the regulation of histone tail methylation. Trithorax group proteins are important mediators of H3K4 di- and trimethylation, marks typically associated with transcriptional activation (Schuettengruber, Chourrout, Vervoort, Leblanc, & Cavalli, 2007). In contrast, polycomb group proteins of the PRC2 complex are responsible for trimethylation of H3K27, a mark of transcriptional inactivation. H3K27me3 further mediates the recruitment of a second polycomb complex, PRC1, which monoubiquitinates histone H2A on lysine 119 and stabilizes the repressive chromatin state (de Napoles et al., 2004; Wang et al., 2004).

2. HEMATOPOIETIC STEM CELL DEVELOPMENT

As for all vertebrate species, the earliest hematopoietic cells in zebrafish represent "primitive" lineages that support embryonic development but are derived from relatively short-lived progenitors that are eventually replaced by HSC-derived cells (de Jong & Zon, 2005). At the molecular level, early HSC development is regulated by a core set of transcription factors including *scl*, *gata2*, and *runx1* (Jagannathan-Bogdan & Zon, 2013). Upstream of these factors, inputs from major signaling pathways including Bmp, Wnt, and Notch contribute to HSC development (Clements & Traver, 2013). In mammals, a major site where HSCs are born is the aorta-gonad-mesonephros region. Within this region, nascent HSCs emerge from a specialized subset of endothelial cells within the dorsal aorta through a process termed the endothelial-to-hematopoietic transition (EHT). During this process, cells of the hemogenic endothelium transition to a hematopoietic fate in the absence of cell division (Bertrand et al., 2010; Boisset et al., 2010; Kissa & Herbomel, 2010). Later, these cells migrate to the fetal liver and eventually to the bone marrow. Evidence suggests that the de novo production of HSCs only occurs during a defined window in the embryo, and self-renewing divisions of this initial pool provide for the expansion and maintenance of HSCs through adulthood (Chen, Yokomizo, Zeigler,

Dzierzak, & Speck, 2009; Göthert et al., 2005). Although more challenging than in vitro approaches, the exquisite spatial and temporal regulation of this process makes the in vivo study of HSC development important.

The rapid development of the hematopoietic system combined with external fertilization and larval clarity make zebrafish an appealing model for the in vivo study of HSC development. In zebrafish, the earliest HSCs emerge from the ventral wall of the dorsal aorta at around 30 h post fertilization (hpf) and HSC production continues until roughly 48 hpf (Bertrand et al., 2010; Kissa & Herbomel, 2010). Exploiting the ability to visualize the process in zebrafish embryos, recent studies identified additional signals required for HSC birth, including Cxcl12 from somite-derived endothelial cells (Nguyen et al., 2014), TNFα from primitive neutrophils (Espín-Palazón et al., 2014), and INF-γ from the hemogenic endothelium itself (Sawamiphak, Kontarakis, & Stainier, 2014). Following emergence, these newly born HSCs rapidly enter the subaortic space and subsequently migrate either directly to the thymus or to the caudal hematopoietic tissue. By 4 dpf, HSCs seed the kidney marrow, which is the site of adult hematopoiesis in zebrafish (Jin, Xu, & Wen, 2007; Murayama et al., 2006).

3. APPROACHES

The dramatic shift in transcriptional profiles that occurs during EHT is likely to require significant changes in the chromatin landscape. However, our current understanding of how epigenetic regulation contributes to the production and early maintenance of HSCs is limited. The accessibility of early stages of HSC development and large brood sizes makes zebrafish well suited to genetic approaches aimed at identifying epigenetic regulators of HSC development. The application of forward and reverse genetic approaches in zebrafish is beginning to unravel the relevant epigenetic pathways. It is important to note that because HSCs are produced from specialized cells within the dorsal aorta, vascular development and artery specification are prerequisites for HSC emergence. In this chapter, we focus our discussion on identified genes that impact early HSC development but do not overtly impair development of the vascular system.

3.1 UNBIASED FORWARD GENETIC APPROACHES

Small size and large brood sizes make zebrafish amenable to unbiased genetic screening approaches. Based on these strengths, several genetic screens have been performed to identify zebrafish mutants with impaired HSC development (Burns et al., 2009; Du et al., 2011; Liu et al., 2015). These screens were not designed to specifically identify epigenetic regulators of HSC development; nonetheless, two known chromatin modifiers were among the identified genes.

Burns et al. (2009) screened a collection of 194 zebrafish lines harboring retroviral insertions in known genes for reduced expression of the HSC marker *c-myb*.

The histone deacetylase enzyme, *hdac1* was identified as one of nine genes in this collection that was required for HSC production. Epistasis analysis demonstrated that *hdac1* is required downstream of Notch signaling and arterial formation. Expression of *runx1*, an essential regulator of EHT, was reduced in *hdac1* mutants and HSCs could be rescued by injection of mRNA encoding this transcription factor. Although direct targets were not identified, these results suggest that *hdac1* acts upstream or in parallel to *runx1* to promote HSC specific transcriptional programs. The identification of *hdac1* in this retroviral screen provided a first indication that epigenetic regulation of chromatin states contributes to the control of HSC emergence.

More recently, an N-ethyl-N-nitrosourea-based screen for genes that disrupt *c-myb* expression identified a requirement for the DNA methyltranserase, *dnmt1*, in the early maintenance of HSCs (Liu et al., 2015). *Dnmt1* mutant larvae had overtly normal vascular development and arterial specification, but exhibited a clear reduction in *c-myb* expression along the dorsal aorta, attributed to a diminished capacity for HSC proliferation in the mutants. Consistent with this hypothesis, *cebpa*, a transcription factor known to negatively regulate HSC proliferation, was upregulated in the mutants, and combined mutation of *cebpa* and *dnmt1* rescued expression of HSC markers. Studies in mouse have also identified a requirement for *Dnmt1* in the self-renewal adult HSCs derived from the bone marrow, suggesting a conserved role for 5mC in HSC maintenance at multiple developmental stages (Bröske et al., 2009; Trowbridge, Snow, Kim, & Orkin, 2009). It remains possible that Dnmt1 also has roles in the de novo generation of HSCs that are masked by maternally deposited mRNA in zebrafish *dnmt1* mutants. Additional analysis of maternal/zygotic *dnmt1* mutant embryos will be required to definitively address requirements for *dnmt1* in early steps of HSC production.

3.2 REVERSE GENETIC APPROACHES

Forward genetic screens provide a powerful approach to identify novel gene functions and pathways. However, they can be labor intensive and screens to date have not been specifically designed to identify epigenetic regulators of HSC development. Reverse genetic approaches for targeted elimination of specific gene products offer an alternative strategy to directly test known epigenetic regulators for their role in HSC development. Reverse genetic approaches also facilitate testing of gene families for functionally overlapping requirements in HSC development. In zebrafish, antisense morpholinos that block translation or splicing of target mRNAs have historically been used as the primary tool for reverse genetics (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009). An advantage of this approach is the ability to rapidly assess gene function, including for more than one gene, in morpholino-injected embryos. However, there has been growing concern over the potential for nonspecific phenotypes in morphant embryos (Kok et al., 2014; Robu et al., 2007). Genome-editing approaches have now emerged as an attractive alternative, allowing for the selective mutation of any gene of interest within the zebrafish genome (Bedell et al., 2012; Hwang et al., 2013).

To date, several groups have provided data implicating polycomb and trithorax group proteins in HSC development using antisense morpholinos. Depletion of the PRC1 genes *Bmi1/1b* or *Ring1b*, led to decreased expression of the HSC markers *runx1* and *c-myb* in the dorsal aorta (Yu et al., 2012). Similarly, morpholino depletion of the trithorax gene *mll* resulted in reduced expression of HSC markers (Wan, Hu, Liu, Feng, & Xiao, 2011). However, rescue of HSCs by coinjection of mRNA encoding wild-type proteins was not demonstrated for any of these morphants and upstream defects in vascular development were not investigated, making it difficult to definitively assess the specificity of these phenotypes. More recently, morpholino-knockdown of the 5mC dioxygenase *tet2* gene in zebrafish was reported to cause reduced expression of the HSC marker *c-myb* (Ge et al., 2014). However, similar reductions in *c-myb* expression were not observed in *tet2* mutant zebrafish, which are viable to adulthood and show no obvious defects in embryonic HSC production (Gjini et al., 2015; Li et al., 2015).

A major advance for the zebrafish field has been the development of tools for the targeted introduction of mutations. Transcription activator-like effector nucleases (TALENs) (Cermak et al., 2011) and clustered, regularly interspaced short palindromic repeats combined with CRISPR-associated proteins (CRISPR/Cas9) (Cong et al., 2013; Mali et al., 2013) can now be used to direct double-strand breaks to specific genomic loci. The subsequent repair of these breaks by the nonhomologous end joining machinery is error-prone and often introduces small insertions or deletions into the targeted gene. Both TALENs and CRISPR/Cas9 systems have been successfully applied in zebrafish with high efficiency (Bedell et al., 2012; Hwang et al., 2013).

Because genome-editing techniques are relatively new to the zebrafish system, they have yet to be extensively exploited for the mutation of known epigenetic regulators. However, the potential of these approaches is illustrated by a recent study identifying overlapping requirements for the 5mC dioxygenases Tet2 and Tet3 in HSC emergence. Using TALENs, Li et al. generated catalytic loss of function alleles for each of the three zebrafish *tet* orthologs and systematically examined all mutant combinations (Li et al., 2015). Mutation of either *tet2* or *tet3* alone caused only modest reductions in 5hmC and was not associated with defective HSC development. However, combinatorial mutation of *tet2* and *tet3* led to a greater than 30-fold reduction in genome-wide 5hmC levels and a significant reduction in the number of successful EHT events observed by time-lapse confocal microscopy (Fig. 1A and B). Mutation of *tet2/3* did not affect vascular development or arterial specification; however, Notch signaling in the hemogenic endothelium and the downstream expression of the *scl/gata2b/runx1* hematopoietic transcription factor network was disrupted in the double mutants (Fig. 1C and D). The results of this study uncover a requirement for Tet enzymes in the function of the hemogenic endothelium and identify dynamic regulation of 5mC as a previously unappreciated aspect of HSC production. The combinatorial requirement for Tets in HSC emergence identified in this study is unlikely to have been detected using forward genetic approaches.

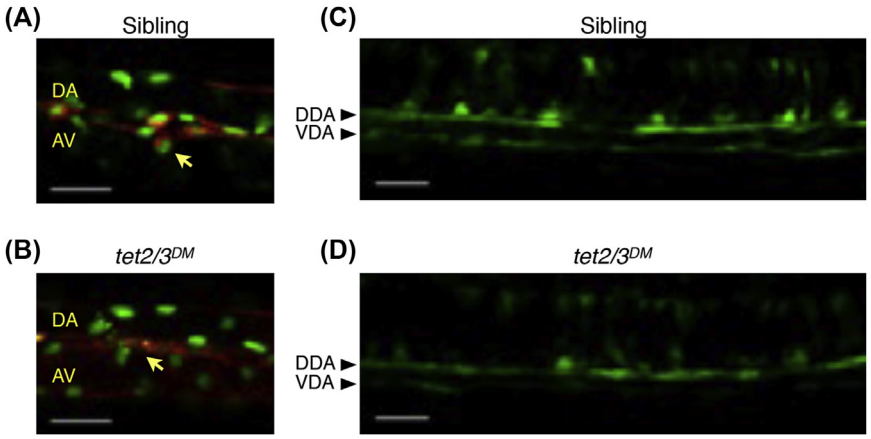

FIGURE 1 Tet2/3 are required for hematopoietic stem cell emergence and Notch signaling in the hemogenic endothelium.

(A, B) Time-lapse confocal images of *Tg*(*kdrl:Ras-mCherry*), *Tg*(*kdrl:H2B-EGFP*) *tet2/3DM* (double mutant), and control siblings from 30 to 46 hpf. The *yellow arrow* indicates a cell that has just completed endothelial-to-hematopoietic transition in a sibling larva (A) and a cell undergoing nuclear fragmentation in a *tet2/3DM* larva (B).
DA indicates the dorsal aorta. *AV* indicates the axial vein. Scale bars indicate 30 μM.
(C, D) Images of *Tg*(*tp1:GFP*) *tet2/3DM* and control siblings at 26 hpf. *DDA* indicates the dorsal wall of the dorsal aorta. *VDA* indicates the ventral wall of the dorsal aorta. Scale bars indicate 60 μM. (See color plate)

3.3 CANDIDATE SCREENS

Reverse genetic approaches provide a straightforward mechanism to evaluate the role of known epigenetic regulators in HSC development. However, this strategy is typically applied to one or a few genes with anticipated phenotypes. Candidate screens essentially leverage advantages of both forward and reverse genetic approaches. A recent candidate screen for chromatin factors that regulate zebrafish hematopoiesis substantially expanded the number of epigenetic regulators implicated in HSC development. Screening of a panel of 425 morpholinos that depleted proteins containing amino acid motifs associated with chromatin or nucleic acid binding revealed 31 candidates that caused strong alterations in *runx1*/*c-myb* expression levels (Huang et al., 2013). Among these candidates, 20 morpholinos impacted HSC development without negative effects on vascular or arterial development (Tables 1 and 2). Consistent with previous studies, this candidate screen implicated *hdac1* and components of the PRC1 and SET1/trithorax complexes in HSC production. In addition, several genes that had not been previously implicated in HSC development were uncovered, including *brd8a*, *jmjd1*, and *nap1l4a*. An added advantage of the large scale—candidate approach is that it allows for identification

Table 1 Epigenetic Regulators With Known Requirements for Hematopoietic Stem Cell Development

Pathway	Gene	Gene Function	Mutant or Morphant	Rescue	References
DNA methylation	*dnmt1*	DNA methyltransferase	Mutant	Yes	Liu et al. (2015)
	tet2	Methylcytosine dioxygenase	Morphant	Yes	Ge et al. (2014)
	tet2; *tet3*	Methylcytosine dioxygenase	Mutant	Yes	Li et al. (2015)
Polycomb regulation	*bmi1*	Polycomb repressive complex 1	Morphant	No	Yu et al. (2012)
	ring1b	Polycomb repressive complex 1	Morphant	No	Yu et al. (2012)
	cbx6b	Polycomb repressive complex 1	Morphant	No	Huang et al. (2013)
	cbx8b	Polycomb repressive complex 1	Morphant	No	Huang et al. (2013)
Histone methylation	*mll*	SET1 histone methyltransferase complex	Morpahnt	No	Wan et al. (2011)
	ash2l	SET1 histone methyltransferase complex	Morphant	No	Huang et al. (2013)
	cxxc1l	SET1 histone methyltransferase complex	Morphant	No	Huang et al. (2013)
	setd1ba	SET1 histone methyltransferase complex	Morphant	No	Huang et al. (2013)
	prdm12	Histone methyltransferase	Morphant	No	Huang et al. (2013)
	prdm16	Histone methyltransferase	Morphant	No	Huang et al. (2013)
Histone deacetylation	*hdac1*	Histone deacetylase	Mutant	No	Burns et al. (2009)
	hdac1	Histone deacetylase	Morphant	No	Huang et al. (2013)
	hdac6	Histone deacetylase	Morphant	No	Huang et al. (2013)
	hdac9a	Histone deacetylase	Morphant	No	Huang et al. (2013)
	sirt7	Histone deacetylase	Morphant	No	Huang et al. (2013)
Chromatin remodeling	*smarcd1*	SWI/SNF chromatin–remodeling complex	Morphant	No	Huang et al. (2013)
	smarcd2	SWI/SNF chromatin–remodeling complex	Morphant	No	Huang et al. (2013)
	nap1l4a	Nucleosome assembly factor	Morphant	No	Huang et al. (2013)
	brd8a	Bromodomain-containing protein	Morphant	No	Huang et al. (2013)
Histone acetylation	*p300*	p300/CBP transcriptional coactivator complex	Morphant	No	Huang et al. (2013)

Table 2 Zebrafish Morphants That Cause an Expansion of Hematopoietic Stem Cell Populations

Pathway	Gene	Gene Function	Mutant or Morphant	Rescue	References
Chromatin remodeling	*cecr2*	Bromodomain-containing protein	Morphant	No	Huang et al. (2013)
	chd7	Chromodomain helicase DNA–binding protein	Morphant	No	Huang et al. (2013)
Histone acetylation	*crebbpa*	CREB-binding protein	Morphant	No	Huang et al. (2013)
Unknown	*mbd6*	Methyl-CpG-binding protein	Morphant	No	Huang et al. (2013)

of multisubunit complexes that include more than one gene implicated by the screen. This analysis revealed likely requirements for the SWI/SNF, ISWI, SET, and HAT complexes in regulating HSC development. The advent and adaptation of Crispr/Cas9 in zebrafish now allows for larger candidate screens that can overcome limitations of high cost and nonspecific effects encountered with morpholinos (Shah, Davey, Whitebirch, Miller, & Moens, 2015). This approach can be used to further evaluate epigenetic components in HSC development.

4. DISEASE MODELS

There is strong evidence that disruption of the chromatin environment contributes to malignancies of the hematopoietic system, including both myeloid (Shih, Abdel-Wahab, Patel, & Levine, 2015) and lymphoid (Jiang & Melnick, 2015) malignancies. Parallels between the hematopoietic system in humans and zebrafish have inspired the use of zebrafish to model human leukemia (White, Rose, & Zon, 2013). In particular, efforts to model myeloid malignancies, a class of clonal diseases arising in hematopoietic stem or progenitor cells, have revealed new insights into how epigenetic mechanisms promote normal and diseased states.

In humans, the *NUP98-HOXA9* (*NHA9*) fusion oncogene is detected in high-risk acute myeloid leukemia. This oncogene results from a chromosomal translocation that fuses nucleoporin 98 kDa (*NUP98*) to homeobox A9 (*HOXA9*) (Nakamura et al., 1996). In zebrafish, expression of the NAH9 fusion protein from a transgenic *pu.1* promoter causes fish to develop myeloproliferative neoplasms in adulthood (Forrester et al., 2011). This same fusion also causes overproduction of HSCs in the zebrafish embryo (Deveau et al., 2015). Microarray analysis revealed that the DNA methyltransferase, *dnmt1*, is one of the most upregulated genes in embryos expressing NHA9 (Deveau et al., 2015). Moreover, chemical inhibition of Dnmt1 by the DNA methyltransferase inhibitor decitabine rescued overproduction of HSCs in embryos carrying the NHA9 transgene, suggesting that Dnmt1 overexpression plays a causative role in promoting HSC expansion. This observation is consistent with the role for *dnmt1* in HSC proliferation that was reported by Liu et al. (2015). Intriguingly, combining sub-monotherapeutic doses of the histone deacetylase inhibitor valproic acid and decitabine also blocked the effects of NHA9 on zebrafish blood development. These results link expression of the NAH9 fusion to changes in the chromatin landscape and highlight the potential of combinatorial epigenetic therapies in the treatment of NHA9-induced myeloid disease.

More recently, the first zebrafish cancer model driven by mutation of a known chromatin regulator was described (Gjini et al., 2015). Mutations in the 5mC dioxygenase *TET2* are detected with high frequencies in human myeloid malignancies and mutations in *Tet2* cause similar malignancies in mouse (Delhommeau et al., 2009; Ko et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). As previously discussed, homozygous mutation of *tet2* does not impact HSC development in the zebrafish embryo. However, as they age, *tet2* mutant zebrafish adults develop

progressive clonal myelodysplasia, culminating in myelodysplastic syndromes by 24 months. Decreases in the modified base 5hmC were observed in hematopoietic cells of the kidney marrow of *tet2* mutants but not in other cell types, suggesting that other Tet family members may compensate for *tet2* outside of the hematopoietic system. The lack of Tet redundancy in HSCs provides one likely explanation for the high oncogenic potential of *TET2* mutation in myeloid malignancies. Because *tet2* mutants are viable and fertile, they provide a powerful model to dissect the pathways that are regulated by Tet2 in the adult hematopoietic system. In addition, *tet2* mutant embryos offer an opportunity for small-molecule screens to identify compounds that selectively kill *tet2* mutant HSCs. Such compounds may have therapeutic potential for the treatment of myeloid malignancies.

5. CURRENT CHALLENGES AND FUTURE DIRECTIONS

To date, genetic approaches in zebrafish have implicated a number of important epigenetic pathways in the regulation of HSC development. However, the generation of additional tools and approaches will be required to fully realize the potential of the zebrafish system.

5.1 TOOLS FOR UNBIASED SCREENING OF EPIGENETIC STATES IN VIVO

To date, screens for regulators of HSC development in zebrafish have relied on expression of HSC associated markers, leading to identification of a broad spectrum of genes with diverse functions. More narrowly focused screens for epigenetic regulators of HSC development have not been performed, likely due to the lack of tools to rapidly monitor epigenetic changes at the enhancers or promoters of relevant hematopoietic genes. Tools to monitor DNA methylation changes in zebrafish using fluorescent reporters under the control of the yeast Gal4/UAS system have previously been described (Akitake, Macurak, Halpern, & Goll, 2011; Goll, Anderson, Stainier, Spradling, & Halpern, 2009). However, the use of exogenous UAS sequences to monitor DNA methylation makes this approach better suited for the identification of global regulators of chromatin, rather than to those involved in a particular developmental process.

A recently described variation on the Gal4/UAS approach has the potential to be adapted for screening in zebrafish. The minimal *Snrpn* gene promoter is a methylation-sensitive promoter that is influenced by the DNA methylation status of adjacent sequences. By placing this promoter upstream of a fluorescent reporter, Stelzer and colleagues were able to visually track methylation changes at nearby enhancer sequences in mouse embryonic stem cells based on fluorophore expression (Stelzer et al., 2015). Placing the Snrpn reporter adjacent to enhancers of hematopoietic transcription factors could provide one approach to monitor the methylation status of HSC-specific enhancers in the developing zebrafish embryo. If successful, this

approach would allow for unbiased screens specifically designed to identify genes involved in the epigenetic control of the HSC transcriptional program.

5.2 TOOLS FOR TISSUE-SPECIFIC MUTATION

Another major challenge is that current approaches have relied on whole embryo depletion of gene products. However, many epigenetic regulators are likely to be important in multiple tissues and mutant embryos may not survive long enough for analysis of HSC development. In other cases, upstream defects in mesoderm patterning, vascular development, or arterial identify may mask later specific requirements for epigenetic regulators in HSC development. These challenges will require the use of tissue-specific approaches for mutation of candidate genes. The Cre/loxP system has been widely used in mouse for the conditional mutation of specific genes (Nagy, 2000). This system is also functional in zebrafish (Hans, Kaslin, Freudenreich, & Brand, 2009; Langenau et al., 2005). A recently described CRISPR/Cas-based vector system that enables tissue-specific gene inactivation in zebrafish provides one exciting alternative that could be applied to test a large number of candidate epigenetic regulators for their requirements in HSCs or their endothelial precursors (Ablain, Durand, Yang, Zhou, & Zon, 2015).

CONCLUSIONS

It is perhaps not surprising that epigenetic regulation plays a major role in HSC generation, as the transition from endothelial to hematopoietic identity represents a dramatic reprogramming of fate. The accessibility of zebrafish embryos from the time of fertilization, coupled with larval clarity and large brood sizes has facilitated the discovery of signals and genes that regulate the generation of HSCs. Identifying mechanisms to manipulate HSC fate and proliferation has important implications for the development of therapeutics to treat hematopoietic disorders. The translational potential of the zebrafish system is exemplified by the development of a PGE2 derivative as a drug to expand transplantable HSCs from human cord blood. A chemical screen identified the capacity of PGE2 for impacting production of zebrafish HSCs (North et al., 2007), and within a few years the concept was being tested in human clinical trials (Hagedorn, Durand, Fast, & Zon, 2014). In addition to chemical screens, and the genetic strategies described above, new gene-editing techniques offer opportunities to build zebrafish models for hematopoietic disease. For example, mutations in genes known to be associated with leukemia, including patient-specific alleles, can be created and combined with *tet2* mutations to investigate the impact on myeloid dysplasia. While the DNA and chromatin modification machineries that are key to HSC biology are now beginning to be defined, the next challenges will be to understand their target genes and to distinguish epigenetic marks that are drivers of HSC phenotype from those that are consequences of altered transcription. Again, emerging technologies provide exciting avenues of inquiry,

limited only by investigators' imagination. For example, modified Crispr/Cas9 technologies can now be developed to target not only mutations, but epigenetic modifications (Hilton et al., 2015; Maeder et al., 2013).

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